

## PATENT COOPERATION TREATY

## PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference TSRI6511	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/US99/11780	International filing date ( <i>day/month/year</i> ) 28 MAY 1999	(Earliest) Priority Date ( <i>day/month/year</i> ) 29 MAY 1999
Applicant THE SCRIPPS RESEARCH INSTITUTE		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. ☐ Certain claims were found unsearchable (See Box I).
2. ☒ Unity of invention is lacking (See Box II).
3. ☐ The international application contains disclosure of a nucleotide and/or amino acid sequence listing and the international search was carried out on the basis of the sequence listing
  - ☐ filed with the international application.
  - ☐ furnished by the applicant separately from the international application,
    - ☐ but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
  - ☐ transcribed by this Authority.
4. With regard to the title,
  - ☒ the text is approved as submitted by the applicant.
  - ☐ the text has been established by this Authority to read as follows:
5. With regard to the abstract,
  - ☒ the text is approved as submitted by the applicant.
  - ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.
6. The figure of the drawings to be published with the abstract is:
  - Figure No. \_\_\_\_\_ ☐ as suggested by the applicant.
  - ☐ because the applicant failed to suggest a figure.
  - ☐ because this figure better characterizes the invention.
  - ☒ None of the figures.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/11780

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-30, 37, 38

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/11780

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 9/00

US CL : 435/183; 514/2

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/183; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MUKHOPADHYAY et al. Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation. Nature. June 1995, Vol. 375, pages 577-581, especially abstract, page 578, col. 2, para. 2 through page 580, and Figs 3 and 4.	1-30 and 38
Y	KAPLAN et al. Association of the amino-terminal half of c-Src with focal adhesions alters their properties and is regulated by phosphorylation of tyrosine 527. EMBO J. 1994, Vol 13, pages 4745-4756, entire document	1-30, 37, 38



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

Date of mailing of the international search report

18 OCT 1999

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No.

Authorized officer  
Richard SchnizerJOYCE BRIDGERS  
PARALEGAL SPECIALIST  
CHEMICAL MATRIX

Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/11780

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MUKHOPADHYAY et al. Wild-type p53 and v-Src exert opposing influences on human vascular endothelial growth factor gene expression. Cancer Research. 15 December 1995, Vol. 55, pages 6161-6165.	1-30, 38
Y	FLORIO et al. Aberrant protein phosphorylation at tyrosine is responsible for the growth inhibitory action of pp60v <sup>src</sup> expressed in the yeast saccharomyces cerevisiae. American Society For Cell Biology. March 1994, Vol. 5, pages 283-296, especially abstract	5-16, 21-30, 38
Y	US 5,264,618 A (FELGNER et al) 23 November 1993, col. 18, lines 18-33; and col. 20, lines 17-25.	14
Y	KOEGL et al. Generation of a temperature-sensitive cSRC. Virology. 1993, Vol. 196, pages 368-371, especially abstract.	1, 3, 12-16, and 37
Y	HIRAI et al. SH2 mutants of c-src that are host dependent for transformation are trans-dominant inhibitors of mouse cell transformation by activated c-src. Genes and Development. December 1990, Vol. 4, No. 12B, pages 2342-2352, abstract only.	1,3,12-16, 37



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/11780

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

### MEDLINE

Src, K295M, Y527F, angiogenesis, (angiogen?(10w)(stimul? or generat? or caus? or improv? or therap?))

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-30, 37 and 38, drawn to a src protein and a method of use.

Group II, claim(s) 1-16, drawn to a nucleic acid encoding a src protein.

Group III, claims 17-32, drawn to and methods of using nucleic acids encoding a src protein.

Group IV, claim 33, drawn to a viral gene transfer vector encoding a src protein.

Group V, claim 34, drawn to a non-viral gene transfer vector encoding a src protein.

Group VI, claim 35, drawn to a viral gene transfer vector encoding a src protein lacking kinase activity.

Group VII, claim 36, drawn to a non-viral gene transfer vector encoding a src protein lacking kinase activity.

The inventions listed as Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Claims 1-16 are drawn to a protein and a nucleic acid. PCT rule 13, 37 C.F.R. 1.475(c,d) does not allow for claims drawn to multiple independent products. If multiple products are claimed, the first invention of the category first mentioned in the claims, and the first recited invention of each of the other categories related to the first invention will be considered as the main invention. See PCT Article 17(3)(a) and 37 C.F.R. 1.476(c).

The technical feature of group I is the Src protein of claim 1. Group I is therefore composed of claims to the Src protein (claims 1-16, 37 and 38), and methods of using it (claims 17-30). The Src protein of claim 1, and genes encoding it, are well known in the art (see for example, Bricknell, Crit Rev Oncog 3: 401-446, 1992, abstract), therefore the claims have no special technical feature.

Groups II-VII are linked to group I because they comprise polynucleotides encoding the protein which is the technical feature of group I. However, because there can be no special technical feature, there is a lack of unity between the groups.

**RECEIVED**

JAN 31 2000

**OLSON & HIERL, LTD.****PCT****PATENT COOPERATION TREATY**

090701500

PCT/US99/11780

**NOTIFICATION OF THE RECORDING  
OF A CHANGE**(PCT Rule 92bis.1 and  
Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

CEPURITIS, Talivaldis  
Olson & Hierl, Ltd.  
36th floor  
20 North Wacker Drive  
Chicago, IL 60606  
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing (day/month/year)

14 January 2000 (14.01.00)

Applicant's or agent's file reference  
tsri6511**IMPORTANT NOTIFICATION**International application No.  
PCT/US99/11780International filing date (day/month/year)  
28 May 1999 (28.05.99)

1. The following indications appeared on record concerning:

☒ the applicant ☐ the inventor ☐ the agent ☐ the common representative

Name and Address

State of Nationality

State of Residence

Telephone No.

Facsimile No.

Teleprinter No.

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☒ the person ☐ the name ☐ the address ☐ the nationality ☐ the residence

Name and Address

THE GOVERNMENT OF THE UNITED  
STATES OF AMERICA  
National Institutes of Health  
Office of Technology Transfer  
Suite 325  
6011 Executive Boulevard  
Rockville, MD 20852  
United States of America

State of Nationality

US

State of Residence

US

Telephone No.

Facsimile No.

Teleprinter No.

3. Further observations, if necessary:  
**Additional applicant**

4. A copy of this notification has been sent to:

☒ the receiving Office ☒ the designated Offices concerned  
☐ the International Searching Authority ☐ the elected Offices concerned  
☐ the International Preliminary Examining Authority ☐ other:The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Authorized officer

R. Raissi

Facsimile No.: (41-22) 740.14.35

Telephone No.: (41-22) 338.83.38

## PATENT COOPERATION TREATY

PCT

NOTIFICATION OF THE RECORDING  
OF A CHANGE(PCT Rule 92bis.1 and  
Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

CEPURITIS, Talivaldis  
Olson & Hierl, Ltd.  
36th floor  
20 North Wacker Drive  
Chicago, IL 60606  
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing (day/month/year) 10 March 2000 (10.03.00)	<b>IMPORTANT NOTIFICATION</b>
Applicant's or agent's file reference tsri6511	
International application No. PCT/US99/11780	International filing date (day/month/year) 28 May 1999 (28.05.99)

1. The following indications appeared on record concerning:

☒ the applicant
 ☒ the inventor
 ☐ the agent
 ☐ the common representative

Name and Address

CHERESH, David, A.  
327 Via Andalusia  
Encinitas, CA 92024  
United States of AmericaState of Nationality  
USState of Residence  
US

Telephone No.

Facsimile No.

Teleprinter No.

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☐ the person
 ☐ the name
 ☒ the address
 ☐ the nationality
 ☐ the residence

Name and Address

CHERESH, David, A.  
3277 Lone Hill Lane  
Encinitas, CA 92024  
United States of AmericaState of Nationality  
USState of Residence  
US

Telephone No.

Facsimile No.

Teleprinter No.

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

☒ the receiving Office
 ☐ the designated Offices concerned  
☐ the International Searching Authority
 ☒ the elected Offices concerned  
☒ the International Preliminary Examining Authority
 ☐ other:
The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Authorized officer

R. Raissi

Facsimile No.: (41-22) 740.14.35

Telephone No.: (41-22) 338.83.38

## PATENT COOPERATION TREATY

PCT

NOTIFICATION OF THE RECORDING  
OF A CHANGE(PCT Rule 92bis.1 and  
Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

RECEIVED

MAR 27 2000

CEPURITIS, Talivaldis OLSON & HIERL, LTD.  
Olson & Hierl, Ltd.  
36th floor  
20 North Wacker Drive  
Chicago, IL 60606  
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing (day/month/year) 10 March 2000 (10.03.00)	<b>IMPORTANT NOTIFICATION</b>
Applicant's or agent's file reference tsri6511	
International application No. PCT/US99/11780	International filing date (day/month/year) 28 May 1999 (28.05.99)

## 1. The following indications appeared on record concerning:

☒ the applicant    ☒ the inventor    ☐ the agent    ☐ the common representative

## Name and Address

ELICEIRI, Brian  
935 Laguna #5  
Carlsbad, CA 92008  
United States of AmericaState of Nationality  
USState of Residence  
US

Telephone No.

Facsimile No.

Teleprinter No.

## 2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☐ the person    ☐ the name    ☒ the address    ☐ the nationality    ☐ the residence

## Name and Address

ELICEIRI, Brian  
3104 Hataca Road  
Carlsbad, CA 92009  
United States of AmericaState of Nationality  
USState of Residence  
US

Telephone No.

Facsimile No.

Teleprinter No.

## 3. Further observations, if necessary:

## 4. A copy of this notification has been sent to:

☒ the receiving Office    ☐ the designated Offices concerned  
☐ the International Searching Authority    ☒ the elected Offices concerned  
☒ the International Preliminary Examining Authority    ☐ other:
The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

R. Raissi

Telephone No.: (41-22) 338.83.38

# PATENT COOPERATION TREATY

090701500

WO 99/61590  
PCT/US99/1178

PCT

## NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

From the INTERNATIONAL BUREAU

To:  
CEPURITIS, Talivaldis  
Olson & Hierl, Ltd.  
36th floor  
20 North Wacker Drive  
Chicago, IL 60606  
ÉTATS-UNIS D'AMÉRIQUE

RECEIVED  
DEC 18 1999  
OLSON & HIERL, I

Date of mailing (day/month/year) 02 December 1999 (02.12.99)		IMPORTANT NOTICE	
Applicant's or agent's file reference tsri6511			
International application No. PCT/US99/11780	International filing date (day/month/year) 28 May 1999 (28.05.99)	Priority date (day/month/year) 29 May 1998 (29.05.98)	
Applicant THE SCRIPPS RESEARCH INSTITUTE et al			

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:  
/ AU,CN,EP,IL,JP,KP,KR,US

In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:  
/ AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CU,CZ,DE,DK,EA,EE,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,UA,UG,UZ,VN,YU,ZA,ZW  
The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on  
02 December 1999 (02.12.99) under No. WO 99/61590

### REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a demand for international preliminary examination must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

### REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

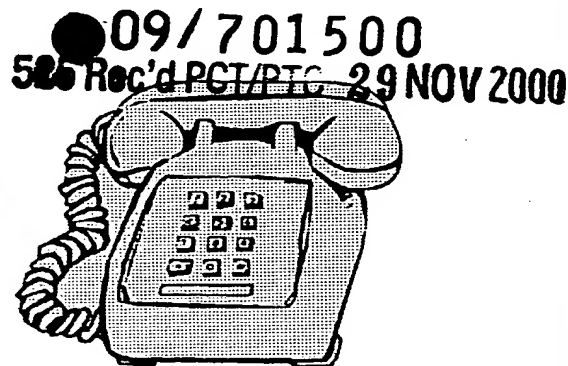
If the applicant wishes to proceed with the international application in the national phase, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. (41-22) 740.14.35	Authorized officer J. Zahra Telephone No. (41-22) 338.83.38
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**CHAPTER I**  
**PCT TELEPHONE MEMORANDUM**  
**FOR**  
**LACK OF UNITY OF INVENTION**

---



PCT No.: PCT/US99/11780

Examiner: Richard Schnizer

Attorney spoken to: TALIVALDIS CEPURITIS

Date of call: 27 JULY 1999

- ☐ Amount of payment approved:
- ☒ Deposit account number to be charged:
- ☐ Attorney elected to pay for ALL additional inventions
- ☐ Attorney elected to pay only for the additional inventions covered by
  - ☐ Group(s):
  - encompassing —
  - ☐ Claim(s):
- ☒ Attorney elected **NOT** to pay for any additional inventions, therefore, only the first claimed invention (Group I) covered by Claim(s) 1-30, 37, 38 has been searched.
- ☒ Attorney was orally advised that there is no right to protest for any group not paid for.
- ☒ Attorney was orally advised that any protest must be filed no later than 15 days from the mailing of the Search Report (PCT/ISA/210).

**Time Limit For Filing A Protest**

Applicant is hereby given 15 days from the mailing date of this Search Report in which to file a protest of the holding of lack of unity of invention. In accordance with PCT Rule 40.2, applicant may protest the holding of lack of unity only with respect to the group(s) paid for.

**Detailed Reasons For Holding Lack Of Unity Of Invention:**

**Detailed Reasons For Holding Lack of Unity Of Invention:**  
*(Continued on a separate sheet)*

**Note: A copy of this form must be attached to the Search Report.**

---

**ATTACHMENT TO CHAPTER I PCT TELEPHONE MEMORANDUM  
FOR  
LACK OF UNITY OF INVENTION**

---

**Detailed Reasons For Holding Lack Of Unity Of Invention:**

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-30, 37 and 38, drawn to a src protein and a method of use.

Group II, claim(s) 1-16, drawn to a nucleic acid encoding a src protein.

Group III, claims 17-32, drawn to and methods of using nucleic acids encoding a src protein.

Group IV, claim 33, drawn to a viral gene transfer vector encoding a src protein.

Group V, claim 34, drawn to a non-viral gene transfer vector encoding a src protein.

Group VI, claim 35, drawn to a viral gene transfer vector encoding a src protein lacking kinase activity.

Group VII, claim 36, drawn to a non-viral gene transfer vector encoding a src protein lacking kinase activity.

The inventions listed as Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Claims 1-16 are drawn to a protein and a nucleic acid. PCT rule 13, 37 C.F.R 1.475(c,d) does not allow for claims drawn to multiple independent products. If multiple products are claimed, the first invention of the category first mentioned in the claims, and the first recited invention of each of the other categories related to the first invention will be considered as the main invention. See PCT Article 17(3)(a) and 37 C.F.R 1.476(c).

The technical feature of group I is the Src protein of claim 1. Group I is therefore composed of claims to the Src protein (claims 1-16, 37 and 38), and methods of using it (claims 17-30). The Src protein of claim 1, and genes encoding it, are well known in the art (see for example, Bricknell, Crit Rev Oncog 3: 401-446, 1992, abstract), therefore the claims have no special technical feature.

Groups II-VII are linked to group I because they comprise polynucleotides encoding the protein which is the technical feature of group I. However, because there can be no special technical feature, there is a lack of unity between the groups.

APR 25 2000

From the  
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

OLSON &amp; HIERL, LTD.

PCT

WRITTEN OPINION

(PCT Rule 66)

To: MARK CHAO  
OLSON & HIERL, LTD.  
20 NORTH WACKER DRIVE  
36TH FLOOR  
CHICAGO IL 60606

ENTERED  
6/25/00  
per

Date of Mailing  
(day/month/year)

20 APR 2000

Applicant's or agent's file reference

TSRI6511

REPLY DUE

within TWO months  
from the above date of mailing

International application No.

PCT/US99/11780

International filing date (day/month/year)

28 MAY 1999

Priority date (day/month/year)

29 MAY 1999

International Patent Classification (IPC) or both national classification and IPC  
IPC(7): C12N 9/00 and US Cl.: 435/183; 514/2

Applicant

THE SCRIPPS RESEARCH INSTITUTE

1. This written opinion is the first (first, etc.) drawn by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

- I ☒ Basis of the opinion
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

3. The applicant is hereby invited to reply to this opinion.

**When?** See the time limit indicated above. ~~The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).~~

**How?** By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

**Also** For an additional opportunity to submit amendments, see Rule 66.4.  
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 *bis*.  
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 29 SEPTEMBER 2001

Name and mailing address of the IPEA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

Richard Schnizer

Telephone No. (703) 308-0196

JOYCE BRIDGERS  
PARALEGAL SPECIALIST  
CHEMICAL MATRIX



WRITTEN OPINION

International application No.

PCT/US99/11780

**I. Basis of the opinion**

1. With regard to the elements of the international application:\*

☒ the international application as originally filed

☒ the description:

pages 1-40 , as originally filed  
pages NONE , filed with the demand  
pages NONE , filed with the letter of

☒ the claims:

pages 41-44 , as originally filed  
pages NONE , as amended (together with any statement) under Article 19  
pages NONE , filed with the demand  
pages NONE , filed with the letter of

☒ the drawings:

pages 1-10 , as originally filed  
pages NONE , filed with the demand  
pages NONE , filed with the letter of

☒ the sequence listing part of the description:

pages NONE , as originally filed  
pages NONE , filed with the demand  
pages NONE , filed with the letter of

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language which is:

- ☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the written opinion was drawn on the basis of the sequence listing:

- ☒ contained in the international application in printed form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

- ☒ the description, pages 1-18
- ☒ the claims, Nos. NONE
- ☒ the drawings, sheets/fig NONE

5. ☐ This opinion has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

\* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".

WRITTEN OPINION

International application No.  
PCT/US99/11780

**III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application.

☒ claims Nos. 31-36

because:

☐ the said international application, or the said claim Nos. \_ relate to the following subject matter which does not require international preliminary examination (*specify*).

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. \_ are so unclear that no meaningful opinion could be formed (*specify*).

☐ the claims, or said claims Nos. \_ are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for said claims Nos. 31-36.

2. A written opinion cannot be drawn due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

WRITTEN OPINION

International application No.

PCT/US99/11780

**V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. statement

Novelty (N)	Claims	<u>1-30, 37, and 38</u>	YES
	Claims	<u>NONE</u>	NO
Inventive Step (IS)	Claims	<u>NONE</u>	YES
	Claims	<u>1-30, 37 and 38</u>	NO
Industrial Applicability (IA)	Claims	<u>1-30, 37, and 38</u>	YES
	Claims	<u>NONE</u>	NO

2. citations and explanations

Claims 1-30, 37 and 38 lack an inventive step under PCT Article 33(3) as being obvious over Mukhopadhyay et al (Nature 375: 577-581, 1995) in view of Kaplan et al (EMBO J. 13(20): 4745-4756, 1994), and Florio et al (Mol. Biol. Cell 5: 283-296, 1994). The invention encompasses compositions comprising active and inactive forms of Src, and methods of using these proteins to stimulate or inhibit angiogenesis. The active and inactive forms of Src are well-known in the art, and their effects on angiogenesis are either known or predictable.

Mukhopadhyay (Nature 375 15: 577-581, 1995) teaches that activation of Src catalytic activity stimulates production of VEGF, a potent stimulator of angiogenesis. Catalytically inactive mutants of Src, which retain the ability to bind Src substrates, can inhibit VEGF production by competing with wild type Src for substrates.

Kaplan teaches that Src normally negatively regulates its activity by autophosphorylation of Y527, and that the mutation Y527F results in a highly active form of Src. Phosphorylation of Y527 normally interferes with substrate recognition. Mutation to Y527F disallows autophosphorylation and results in a form of Src which constitutively recognizes and phosphorylates substrates. Kaplan also teaches the first 251 amino acids of Src are required for appropriate cellular localization but that truncated versions comprising these amino acids (such as Src 251) are catalytically inactive.

Florio teaches that Src K295M is catalytically inactive.

Felgner teaches that liposomes can be used to deliver proteins in vivo, and suggests that liposome/protein complexes can be used to treat disease.

It would have been obvious to one of ordinary skill in the art to modulate angiogenesis in tissue by administration of active or inactive forms of Src. One would have been motivated to do so because Mukhopadhyay teaches that active Src can stimulate angiogenesis by stimulating production of VEGF, and that inactive forms of Src can inhibit expression of VEGF. One would have been motivated to deliver Src in a liposomal complex because Felgner suggests that such complexes (Continued on Supplemental Sheet.)

WRITTEN OPINION

International application No.

PCT/US99/11780

**Supplemental Box**

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

**TIME LIMIT:**

The time limit set for response to a Written Opinion may not be extended. 37 CFR 1.484(d). Any response received after the expiration of the time limit set in the Written Opinion will not be considered in preparing the International Preliminary Examination Report.

**V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):**  
can deliver proteins to cells for therapeutic benefit.

\_\_\_\_\_ NEW CITATIONS \_\_\_\_\_

NONE

## PATENT COOPERATION TREATY

## PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

16

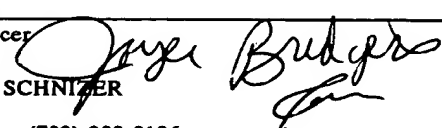
Applicant's or agent's file reference TSRI6511	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US99/11780	International filing date (day/month/year) 28 MAY 1999	Priority date (day/month/year) 29 MAY 1998
International Patent Classification (IPC) or national classification and IPC IPC(7): C12N 9/00 and US Cl.: 435/183; 514/2		
Applicant THE SCRIPPS RESEARCH INSTITUTE		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 5 sheets.
- ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand 15 DECEMBER 1999	Date of completion of this report 24 JULY 2000
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer  RICHARD SCHNITZER
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/11780

**I. Basis of the report****1. With regard to the elements of the international application:\***☒ the international application as originally filed☒ the description:

pages 1-40, as originally filed  
pages NONE, filed with the demand  
pages NONE, filed with the letter of \_\_\_\_\_

☒ the claims:

pages 41-44, as originally filed  
pages NONE, as amended (together with any statement) under Article 19  
pages NONE, filed with the demand  
pages NONE, filed with the letter of \_\_\_\_\_

☒ the drawings:

pages 1-10, as originally filed  
pages NONE, filed with the demand  
pages NONE, filed with the letter of \_\_\_\_\_

☒ the sequence listing part of the description:

pages 1-18, as originally filed  
pages NONE, filed with the demand  
pages NONE, filed with the letter of \_\_\_\_\_

**2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.**

These elements were available or furnished to this Authority in the following language \_\_\_\_\_ which is:

- ☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).  
☐ the language of publication of the international application (under Rule 48.3(b)).  
☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

**3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:**

- ☒ contained in the international application in printed form.  
☒ filed together with the international application in computer readable form.  
☐ furnished subsequently to this Authority in written form.  
☐ furnished subsequently to this Authority in computer readable form.  
☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.  
☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

**4. ☒ The amendments have resulted in the cancellation of:**

- ☒ the description, pages \_\_\_\_\_  
☒ the claims, Nos. NONE  
☒ the drawings, sheets/fig NONE

**5. ☒ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).\*\***

\* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).

\*\*Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.  
PCT/US99/11780

## III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application.

☒ claims Nos. 31-36

because:

☐ the said international application, or the said claim Nos. \_ relate to the following subject matter which does not require international preliminary examination (*specify*).

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. \_ are so unclear that no meaningful opinion could be formed (*specify*).

☐ the claims, or said claims Nos. \_ are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for said claims Nos. 31-36.

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/11780

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement****1. statement**

Novelty (N)

Claims	<u>1-30, 37, and 38</u>	YES
Claims	<u>NONE</u>	NO

Inventive Step (IS)

Claims	<u>NONE</u>	YES
Claims	<u>1-30, 37 and 38</u>	NO

Industrial Applicability (IA)

Claims	<u>1-30, 37, and 38</u>	YES
Claims	<u>NONE</u>	NO

**2. citations and explanations (Rule 70.7)**

Claims 1-30, 37 and 38 lack an inventive step under PCT Article 33(3) as being obvious over Mukhopadhyay et al (Nature 375: 577-581, 1995) in view of Kaplan et al (EMBO J. 13(20): 4745-4756, 1994), and Florio et al (Mol. Biol. Cell 5: 283-296, 1994). The invention encompasses compositions comprising active and inactive forms of Src, and methods of using these proteins to stimulate or inhibit angiogenesis. The active and inactive forms of Src are well-known in the art, and their effects on angiogenesis are either known or predictable.

Mukhopadhyay (Nature 375 15: 577-581, 1995) teaches that activation of Src catalytic activity stimulates production of VEGF, a potent stimulator of angiogenesis. Catalytically inactive mutants of Src, which retain the ability to bind Src substrates, can inhibit VEGF production by competing with wild type Src for substrates.

Kaplan teaches that Src normally negatively regulates its activity by autophosphorylation of Y527, and that the mutation Y527F results in a highly active form of Src. Phosphorylation of Y527 normally interferes with substrate recognition. Mutation to Y527F disallows autophosphorylation and results in a form of Src which constitutively recognizes and phosphorylates substrates. Kaplan also teaches the first 251 amino acids of Src are required for appropriate cellular localization but that truncated versions comprising these amino acids (such as Src 251) are catalytically inactive.

Florio teaches that Src K295M is catalytically inactive.

Felgner teaches that liposomes can be used to deliver proteins in vivo, and suggests that liposome/protein complexes can be used to treat disease.

It would have been obvious to one of ordinary skill in the art to modulate angiogenesis in tissue by administration of active or inactive forms of Src. One would have been motivated to do so because Mukhopadhyay teaches that active Src can stimulate angiogenesis by stimulating production of VEGF, and that inactive forms of Src can inhibit expression of VEGF. One would have been motivated to deliver Src in a liposomal complex because Felgner suggests that such complexes (Continued on Supplemental Sheet.)



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/11780

**Supplemental Box**

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

**I. BASIS OF REPORT:**

5. (Some) amendments are considered to go beyond the disclosure as filed:  
NONE

**V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):**  
can deliver proteins to cells for therapeutic benefit.

----- NEW CITATIONS -----

NONE



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : C12N 9/00	A1	(11) International Publication Number: <b>WO 99/61590</b> (43) International Publication Date: 2 December 1999 (02.12.99)
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(21) International Application Number: PCT/US99/11780

(22) International Filing Date: 28 May 1999 (28.05.99)

(30) Priority Data:  
60/087,220 29 May 1998 (29.05.98) US(71) Applicant (for all designated States except US): THE SCRIPPS  
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(72) Inventors; and

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National Institute of Health, Bethesda, MD 20892-4472  
(US).(74) Agents: CEPURITIS, Talivaldis et al.; Olson & Hierl, Ltd.,  
36th floor, 20 North Wacker Drive, Chicago, IL 60606 (US).(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG,  
BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB,  
GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG,  
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SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA,  
ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ,  
UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD,  
RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK,  
ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI  
patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR,  
NE, SN, TD, TG).**Published***With international search report.**Before the expiration of the time limit for amending the  
claims and to be republished in the event of the receipt of  
amendments.*

(54) Title: METHODS AND COMPOSITIONS USEFUL FOR MODULATION OF ANGIOGENESIS USING TYROSINE KINASE SRC

## (57) Abstract

The present invention describes methods for modulating angiogenesis in tissues using Src protein, modified Src protein, and nucleic acids encoding for such. Particularly the invention describes methods for inhibiting angiogenesis using an inactive Src protein, or nucleic acids encoding therefor, or for potentiating angiogenesis using an active Src protein, or nucleic acids encoding therefor. The invention also describes the use of gene delivery systems for providing nucleic acids encoding for the Src protein, or modified forms thereof.

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EE	Estonia						

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/11780

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 9/00

US CL : 435/183; 514/2

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/183; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MUKHOPADHYAY et al. Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation. Nature. June 1995, Vol. 375, pages 577-581, especially abstract, page 578, col. 2, para. 2 through page 580, and Figs 3 and 4.	1-30 and 38
Y	KAPLAN et al. Association of the amino-terminal half of c-Src with focal adhesions alters their properties and is regulated by phosphorylation of tyrosine 527. EMBO J. 1994, Vol 13, pages 4745-4756, entire document	1-30, 37, 38

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

Date of mailing of the international search report

18 OCT 1999

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No.

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Richard SchnitzerJOYCE BRIDGERS  
PARALEGAL SPECIALIST  
CHEMICAL MATRIS

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/11780

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MUKHOPADHYAY et al. Wild-type p53 and v-Src exert opposing influences on human vascular endothelial growth factor gene expression. Cancer Research. 15 December 1995, Vol. 55, pages 6161-6165.	1-30, 38
Y	FLORIO et al. Aberrant protein phosphorylation at tyrosine is responsible for the growth inhibitory action of pp60v <sup>src</sup> expressed in the yeast saccharomyces cerevisiae. American Society For Cell Biology. March 1994, Vol. 5, pages 283-296, especially abstract	5-16, 21-30, 38
Y	US 5,264,618 A (FELGNER et al) 23 November 1993, col. 18, lines 18-33; and col. 20, lines 17-25.	14
Y	KOEGL et al. Generation of a temperature-sensitive cSRC. Virology. 1993, Vol. 196, pages 368-371, especially abstract.	1, 3, 12-16, and 37
Y	HIRAI et al. SH2 mutants of c-src that are host dependent for transformation are trans-dominant inhibitors of mouse cell transformation by activated c-src. Genes and Development. December 1990, Vol. 4, No. 12B, pages 2342-2352, abstract only.	1,3,12-16, 37

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/11780

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-30, 37, 38

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/11780

### B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

#### MEDLINE

Src, K295M, Y527F, angiogenesis, (angiogen?(10w)(stimul? or generat? or caus? or improv? or therap?))

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-30, 37 and 38, drawn to a src protein and a method of use.

Group II, claim(s) 1-16, drawn to a nucleic acid encoding a src protein.

Group III, claims 17-32, drawn to and methods of using nucleic acids encoding a src protein.

Group IV, claim 33, drawn to a viral gene transfer vector encoding a src protein.

Group V, claim 34, drawn to a non-viral gene transfer vector encoding a src protein.

Group VI, claim 35, drawn to a viral gene transfer vector encoding a src protein lacking kinase activity.

Group VII, claim 36, drawn to a non-viral gene transfer vector encoding a src protein lacking kinase activity.

The inventions listed as Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Claims 1-16 are drawn to a protein and a nucleic acid. PCT rule 13, 37 C.F.R. 1.475(c,d) does not allow for claims drawn to multiple independent products. If multiple products are claimed, the first invention of the category first mentioned in the claims, and the first recited invention of each of the other categories related to the first invention will be considered as the main invention. See PCT Article 17(3)(a) and 37 C.F.R. 1.476(c).

The technical feature of group I is the Src protein of claim 1. Group I is therefore composed of claims to the Src protein (claims 1-16, 37 and 38), and methods of using it (claims 17-30). The Src protein of claim 1, and genes encoding it, are well known in the art (see for example, Bricknell, Crit Rev Oncog 3: 401-446, 1992, abstract), therefore the claims have no special technical feature.

Groups II-VII are linked to group I because they comprise polynucleotides encoding the protein which is the technical feature of group I. However, because there can be no special technical feature, there is a lack of unity between the groups.

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## METHODS AND COMPOSITIONS USEFUL FOR MODULATION OF ANGIOGENESIS USING TYROSINE KINASE SRC

### Reference to Related Application

5        This application claims the benefit of U.S. Provisional Application Serial No. 60/087,220 filed May 29, 1998, which is incorporated by reference, as are all references cited herein.

### Technical Field

10       The present invention relates generally to the field of medicine, and relates specifically to methods and compositions for modulating angiogenesis of tissues using the protein tyrosine kinase Src, variants of Src, and nucleic acids encoding them.

### Background

15       Angiogenesis is a process of tissue vascularization that involves the growth of new developing blood vessels into a tissue, and is also referred to as neo-vascularization. The process is mediated by the infiltration of endothelial cells and smooth muscle cells. The process is believed to proceed in any one of three ways: the vessels can sprout from pre-existing vessels, de-novo development of vessels can arise from precursor cells (vasculogenesis), or existing small vessels can  
20       enlarge in diameter. Blood et al., Bioch. Biophys. Acta, 1032:89-118 (1990).

      Angiogenesis is an important process in neonatal growth, but is also important in wound healing and in the pathogenesis of a large variety of clinical diseases including tissue inflammation, arthritis, tumor growth, diabetic retinopathy, macular degeneration by neovascularization of the retina and like conditions.  
25       These clinical manifestations associated with angiogenesis are referred to as angiogenic diseases. Folkman et al., Science, 235:442-447 (1987). Angiogenesis is generally absent in adult or mature tissues, although it does occur in wound healing and in the corpus luteum growth cycle. See, for example, Moses et al., Science, 248:1408-1410 (1990).

30       It has been proposed that inhibition of angiogenesis would be a useful therapy for restricting tumor growth. Inhibition of angiogenesis has been proposed by (1) inhibition of release of "angiogenic molecules" such as bFGF (basic fibroblast growth factor), (2) neutralization of angiogenic molecules, such as by use of anti-

$\beta$ bFGF antibodies, (3) use of inhibitors of vitronectin receptor  $\alpha_v\beta_3$ , and (4) inhibition of endothelial cell response to angiogenic stimuli. This latter strategy has received attention, and Folkman et al., Cancer Biology, 3:89-96 (1992), have described several endothelial cell response inhibitors, including collagenase inhibitor, basement membrane turnover inhibitors, angiostatic steroids, fungal-derived angiogenesis inhibitors, platelet factor 4, thrombospondin, arthritis drugs such as D-penicillamine and gold thiomalate, vitamin D<sub>3</sub> analogs, alpha-interferon, and the like that might be used to inhibit angiogenesis. For additional proposed inhibitors of angiogenesis, see Blood et al., Bioch. Biophys. Acta., 1032:89-118 (1990), Moses et al., Science, 248:1408-1410 (1990), Ingber et al., Lab. Invest., 59:44-51 (1988), and United States Patent Nos. 5,092,885, 5,112,946, 5,192,744, 5,202,352, 5,753,230 and 5,766,591. None of the inhibitors of angiogenesis described in the foregoing references involve the Src proteins.

For angiogenesis to occur, endothelial cells must first degrade and cross the blood vessel basement membrane in a similar manner used by tumor cells during invasion and metastasis formation.

It has been previously reported that angiogenesis depends on the interaction between vascular integrins and extracellular matrix proteins. Brooks et al., Science, 264:569-571 (1994). Furthermore, it was reported that programmed cell death (apoptosis) of angiogenic vascular cells is initiated by the interaction, which would be inhibited by certain antagonists of the vascular integrin  $\alpha_v\beta_3$ . Brooks et al., Cell, 79:1157-1164 (1994). More recently, it has been reported that the binding of matrix metalloproteinase-2 (MMP-2) to vitronectin receptor ( $\alpha_v\beta_5$ ) can be inhibited using  $\alpha_v\beta_5$  antagonists, and thereby inhibit the enzymatic function of the proteinase. Brooks et al., Cell, 85:683-693 (1996).

#### Summary of the Invention

The present invention is directed to modulation of angiogenesis in tissues by tyrosine kinase Src, also referred to generically herein as Src.

Compositions and methods for modulating angiogenesis in a tissue associated with a disease condition are contemplated. A composition comprising an angiogenesis-modulating amount of a Src protein is administered to tissue to be treated for a disease condition that responds to modulation of angiogenesis. The composition providing the Src protein can contain purified protein, biologically

active protein fragments, recombinantly produced Src protein or protein fragments or fusion proteins thereof, or gene/nucleic acid expression vectors for expressing a Src protein.

5 Where the Src protein is inactivated or inhibited, the modulation is an inhibition of angiogenesis. Where the Src protein is active or activated, the modulation is a potentiation of angiogenesis.

The tissue to be treated can be any tissue in which modulation of angiogenesis is desirable. For angiogenesis inhibition, it is useful to treat diseased tissue where deleterious neovascularization is occurring. Exemplary tissues include inflamed  
10 tissue, solid tumors, metastases, tissues undergoing restenosis, and the like tissues.

For potentiation, it is useful to treat patients with ischemic limbs in which there is poor circulation in the limbs from diabetic or other conditions. Patients with chronic wounds that do not heal and therefore could benefit from the increase in vascular cell proliferation and neovascularization can be treated as well.

15 Particularly preferred is the use of Src protein containing a modified amino acid sequence as described herein. Several particularly useful modified Src proteins and the expression thereof are described herein.

The present invention also encompasses a pharmaceutical composition for stimulating angiogenesis in a target mammalian tissue comprising a viral or non-  
20 viral gene transfer vector containing a nucleic acid and a pharmaceutically acceptable carrier or excipient; said nucleic acid having a nucleic acid segment encoding for a src protein, said src protein having any amino acid residue at codon 527 except tyrosine, serine or threonine.

Also envisioned is a pharmaceutical composition for inhibiting angiogenesis in  
25 a target mammalian tissue comprising a viral or non-viral gene transfer vector containing a nucleic acid and a pharmaceutically acceptable carrier or excipient; said nucleic acid having a nucleic acid segment encoding for a src protein having no kinase activity.

#### Brief Description of the Drawings

30 In the drawings forming a portion of this disclosure:

FIG. 1 is a cDNA sequence of chicken c-Src which is the complete coding sequence with the introns deleted as first described by Takeya et al., Cell, 32:881-890 (1983). The sequence is accessible through GenBank Accession Number

J00844. The sequence contains 1759 nucleotides with the protein coding portion beginning and ending at the respective nucleotide positions 112 and 1713.

FIG. 2 is the encoded amino acid residue sequence of chicken c-Src of the coding sequence shown in FIG. 1.

5        FIG. 3 is a cDNA sequence of human c-Src which as first described by Braeuninger et al., Proc. Natl. Acad. Sci., USA, 88:10411-10415 (1991). The sequence is accessible through GenBank Accession Number X59932 X71157. The sequence contains 2187 nucleotides with the protein coding portion beginning and ending at the respective nucleotide positions 134 and 1486.

10        FIG. 4 is the encoded amino acid residue sequence of human c-Src of the coding sequence shown in FIG. 3.

FIG. 5 illustrates the activation of endogenous Src by bFGF or VEGF as described in Example 4. The top portion of the figure indicates the results of an in vitro kinase assay with the fold activation of endogenous c-Src by either bFGF and VEGF. The bottom portion of the figure is the kinase assay blot probed with an anti-Src antibody as a loading control for equivalent Src and IgG content.

15        FIG. 6 illustrates the effect of retrovirus-mediated gene expression of c-Src A on angiogenesis in the chick chorioallantoic membrane (CAM) as described in Example 4. Nine-day-old chick CAMs were exposed to RCAS-Src A (active mutated c-Src) or control RCAS-GFP (Green Fluorescent Protein; a fluorescent indicator protein) retroviruses or buffer for 72 h. The level of angiogenesis was quantified as shown in FIG. 6A with representative photomicrographs (4x) in FIG. 6B corresponding to each treatment taken with a stereomicroscope.

20        FIG. 7 illustrates the retroviral expression of c-Src A in activating vascular MAP kinase phosphorylation. FIG. 7A shows tissue extracts of 10 day-old chick CAMs that had been exposed to VEGF or PMA for 30 minutes or infected with c-Src A retrovirus for 48 hours. NT stands for no treatment. Src was immunoprecipitated from equivalent amounts of total protein extract and subjected to an in vitro immune complex kinase assay using a FAK-GST fusion protein as a substrate, electrophoresed and transferred to nitrocellulose. Aliquots of the above whole tissue lysates were also measured for endogenous ERK phosphorylation by immunoblotting with an anti-phospho-ERK antibody. FIG. 7B shows 10 day old CAMs that were infected with either mock RCAS or RCAS containing SRC A.

After two days, CAMs were dissected, cryopreserved in OCT and sectioned at 4  $\mu$ m. Sections were immunostained with an anti-phosphorylated ERK antibody (New England Biolabs), washed and detected with a goat anti-rabbit FITC-conjugated secondary antibody. Florescent images were captured on a cooled-  
5 CCD camera (Princeton Inst.)

FIG. 8 illustrates the selective requirement for Src activity during VEGF, but not bFGF-induced angiogenesis. Nine day old chick CAMs were exposed to RCAS-Src 251 or control RCAS-GFP retroviruses or buffer for 20 hours and then incubated for an additional 72 hours in the presence or absence of bFGF or  
10 VEGF. The level of angiogenesis was quantified FIG. 8A as described above, and representative photomicrographs (6x) were taken with a stereomicroscope as shown in FIG. 8B. FIG. 8C shows a blot probed with an anti-Src antibody to confirm the expression of Src 251 in transfected cells as compared to mock treatments.

FIG. 9 illustrates the results of retroviral delivery of RCAS-Src 251 to human tumors. FIG. 9A is a micrograph that shows human medulloblastoma tumor fragment infected with RCAS-GFP (RCAS-Green Fluorescent Protein) expressing GFP exclusively in the tumor blood vessels (arrowhead) as detected by optical sectioning with a Bio Rad laser confocal scanning microscope (bar=500  $\mu$ m).  
20 FIG. 9B depicts data from tumors treated with topical application of retrovirus, which were allowed to grow for 3 or 6 days after which they were resected and wet weights determined. Data are expressed as the mean change in tumor weight (from the 50 mg tumor starting weight) +/- SEM of 2 replicates. FIG. 9C depicts in representative micrographs, medulloblastoma tumors surgically removed from  
25 the embryos (bar=350  $\mu$ m). The lower panels are high magnification views of each tumor showing the vasculature of each tumor in detail (bar=350  $\mu$ m). The arrowhead indicates blood vessel disruption in RCAS-Src251-treated tumors.

FIG. 10 is a diagram illustrating a restriction map of the RCASBP (RCAS) vector construct.

## 30 Detailed Description of the Invention

### A. Definitions

Amino Acid Residue: An amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues

described herein are preferably in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH<sub>2</sub> refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide in keeping with standard polypeptide nomenclature (described in J. Biol. Chem., 243:3552-59 (1969) and adopted at 37 CFR §1.822(b)(2)).

It should be noted that all amino acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues.

Polypeptide: refers to a linear array of amino acid residues connected to one another by peptide bonds between the alpha-amino group and carboxy group of contiguous amino acid residues.

Peptide: as used herein refers to a linear array of no more than about 50 amino acid residues connected one to the other as in a polypeptide.

Cyclic peptide: refers to a compound having a ring structure that includes several amide bonds as in a typical peptide. The cyclic peptide can be a "head to tail" homodetic cyclic peptide, or it can contain a heterodetic ring structure in which the ring is closed by disulfide bridges, lactam bridges, thioesters, thioamides, guanidino, and the like linkages.

Protein: refers to a linear array of more than 50 amino acid residues connected one to the other as in a polypeptide.

Fusion protein: refers to a polypeptide containing at least two different polypeptide domains operatively linked by a typical peptide bond ("fused"), where the two domains correspond to peptides not found fused in nature.

Synthetic peptide: refers to a chemically produced chain of amino acid residues linked together by peptide bonds that is free of naturally occurring proteins and fragments thereof.

#### B. General Considerations

The present invention relates generally to the discovery that angiogenesis is mediated by the tyrosine kinase Src protein, and that angiogenesis can be

modulated by providing either active or inactive Src proteins for potentiating or inhibiting angiogenesis, respectively.

5 This discovery is important because of the role that angiogenesis, the formation of new blood vessels, plays in a variety of disease processes. Where tissues associated with a disease condition require angiogenesis for tissue growth, it is desirable to inhibit angiogenesis and thereby inhibit the diseased tissue growth. Where injured tissue requires angiogenesis for tissue growth and healing, it is desirable to potentiate or promote angiogenesis and thereby promote tissue healing and growth.

10 Where the growth of new blood vessels is the cause of, or contributes to, the pathology associated with a diseased tissue, inhibition of angiogenesis reduces the deleterious effects of the disease. By inhibiting angiogenesis, one can intervene in the disease, ameliorate the symptoms, and in some cases cure the disease.

15 Examples of tissue associated with disease and neovascularization that will benefit from inhibitory modulation of angiogenesis include rheumatoid arthritis, diabetic retinopathy, inflammatory diseases, restenosis, and the like. Where the growth of new blood vessels is required to support growth of a deleterious tissue, inhibition of angiogenesis will reduce the blood supply to the tissue and thereby contribute to reduction in tissue mass based on blood supply requirements.

20 Examples include growth of tumors where neovascularization is a continual requirement in order that the tumor grow beyond a few millimeters in thickness, and for the establishment of solid tumor metastases.

25 Where the growth of new blood vessels contributes to healing of tissue, potentiation of angiogenesis assists in healing. Examples include treatment of patients with ischemic limbs in which there is poor circulation in the limbs from diabetes or other conditions. Also contemplated for treatment are patients with chronic wounds that do not heal and therefore could benefit from the increase in vascular cell proliferation and neovascularization.

30 The methods of the present invention are effective in part because the therapy is highly selective for angiogenesis and not other biological processes.

As described earlier, angiogenesis includes a variety of processes involving neovascularization of a tissue including "sprouting", vasculogenesis, or vessel enlargement, all of which angiogenesis processes are effected by Src protein.

With the exception of traumatic wound healing, corpus luteum formation and embryogenesis, it is believed that the majority of angiogenesis processes are associated with disease processes. Accordingly, the present therapeutic methods are selective for the disease and do not have deleterious side effects.

5 C. Src Proteins

A tyrosine kinase Src protein for use in the present invention can vary depending upon the intended use. The terms "Src protein" or "Src" are used to refer to the various forms of tyrosine kinase Src proteins described herein, either in active or inactive forms.

10 An "active Src protein" refers to any of a variety of forms of src protein which potentiate angiogenesis. Assays to measure potentiation of angiogenesis are described herein, and are not to be construed as limiting. A protein is considered active if the level of angiogenesis is at least 10% greater, preferably 25% greater, and more preferably 50% greater than a control level where no src is added to the  
15 assay system. The preferred assay for measuring potentiation is the CAM assay using RCAS viral vector as described in the Examples in which the angiogenic index is calculated by counting branch points. A preferred active Src protein exhibits tyrosine kinase activity as well. Exemplary active Src proteins are described in the Examples, and include Src-A.

20 An "inactive Src protein" refers to any of a variety of forms of Src protein which inhibit angiogenesis. Assays to measure inhibition of angiogenesis are described herein, and are not to be construed as limiting. A protein is considered inactive if the level of angiogenesis is at least 10% lower, preferably 25% lower, and more preferably 50% lower than a control level where no exogenous Src is  
25 added to the assay system. The preferred assay for measuring inhibition is the CAM assay using RCAS viral vector as described in the Examples in which the angiogenic index is calculated by counting branch points. A preferred inactive Src protein exhibits reduced tyrosine kinase activity as well. Exemplary inactive Src proteins are described in the Examples, and include Src-251.

30 A Src protein useful in the present invention can be produced in any of a variety of methods including isolation from natural sources including tissue, production by recombinant DNA expression and purification, and the like. Src



protein can also be provided "in situ" by introduction of a gene therapy system to the tissue of interest which then expresses the protein in the tissue.

A gene encoding a Src protein can be prepared by a variety of methods known in the art, and the invention is not to be construed as limiting in this regard. For example, the natural history of Src is well known to include a variety of homologs from mammalian, avian, viral and the like species, and the gene can readily be cloned using cDNA cloning methods from any tissue expressing the protein. A preferred Src for use in the invention is a cellular protein, such as the mammalian or avian homologs designated c-Src. Particularly preferred is human c-Src.

10 D. Recombinant DNA Molecules and Expression Systems for Expression of a Src Protein

The invention describes several nucleotide sequences of particular use in the present invention. These sequences include sequences which encode a Src protein useful in the invention, and various DNA segments, recombinant DNA (rDNA) molecules and vectors constructed for expression of Src protein.

15 DNA molecules (segments) of this invention therefore can comprise sequences which encode whole structural genes, fragments of structural genes, and transcription units as described further herein.

A preferred DNA segment is a nucleotide sequence which encodes a Src protein as defined herein, or biologically active fragment thereof,

20 The amino acid residue sequence and nucleotide sequence of a preferred c-Src is described in the Examples.

A preferred DNA segment codes for an amino acid residue sequence substantially the same as, and preferably consisting essentially of, an amino acid residue sequence or portions thereof corresponding to a Src protein described herein. Representative and preferred DNA segments are further described in the Examples.

25 The amino acid residue sequence of a protein or polypeptide is directly related via the genetic code to the deoxyribonucleic acid (DNA) sequence of the structural gene that codes for the protein. Thus, a structural gene or DNA segment can be defined in terms of the amino acid residue sequence, i.e., protein or polypeptide, for which it codes.

30

An important and well known feature of the genetic code is its redundancy. That is, for most of the amino acids used to make proteins, more than one coding nucleotide triplet (codon) can code for or designate a particular amino acid residue. Therefore, a number of different nucleotide sequences may code for a particular amino acid residue sequence. Such nucleotide sequences are considered functionally equivalent since they can result in the production of the same amino acid residue sequence in all organisms. Occasionally, a methylated variant of a purine or pyrimidine may be incorporated into a given nucleotide sequence. However, such methylations do not affect the coding relationship in any way.

A nucleic acid is any polynucleotide or nucleic acid fragment, whether it be a polyribonucleotide or polydeoxyribonucleotide, i.e., RNA or DNA, or analogs thereof. In preferred embodiments, a nucleic acid molecule is in the form of a segment of duplex DNA, i.e., a DNA segment, although for certain molecular biological methodologies, single-stranded DNA or RNA is preferred.

DNA segments are produced by a number of means including chemical synthesis methods and recombinant approaches, preferably by cloning or by polymerase chain reaction (PCR). DNA segments that encode portions of a Src protein can easily be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci et al, *J. Am. Chem. Soc.*, 103:3185-3191, 1981, or using automated synthesis methods. In addition, larger DNA segments can readily be prepared by well known methods, such as synthesis of a group of oligonucleotides that define the DNA segment, followed by hybridization and ligation of oligonucleotides to build the complete segment. Alternative methods include isolation of a preferred DNA segment by PCR with a pair of oligonucleotide primers used on a cDNA library believed to contain members which encode a Src protein.

Of course, through chemical synthesis, any desired modifications can be made simply by substituting the appropriate bases for those encoding the native amino acid residue sequence. This method is well known, and can be readily applied to the production of the various different "modified" Src proteins described herein.

Furthermore, DNA segments consisting essentially of structural genes encoding a Src protein can be subsequently modified, as by site-directed or random mutagenesis, to introduce any desired substitutions.

### 1. Cloning a Src Gene

A Src gene can be cloned from a suitable source of genomic DNA or messenger RNA (mRNA) by a variety of biochemical methods. Cloning of these genes can be conducted according to the general methods described in the Examples and as known in the art.

Sources of nucleic acids for cloning a Src gene suitable for use in the methods of this invention can include genomic DNA or messenger RNA (mRNA) in the form of a cDNA library, from a tissue believed to express these proteins. A preferred tissue is human lung tissue, although any other suitable tissue may be used.

A preferred cloning method involves the preparation of a cDNA library using standard methods, and isolating the Src-encoding nucleotide sequence by PCR amplification using paired oligonucleotide primers based on the nucleotide sequences described herein. Alternatively, the desired cDNA clones can be identified and isolated from a cDNA or genomic library by conventional nucleic acid hybridization methods using a hybridization probe based on the nucleic acid sequences described herein. Other methods of isolating and cloning suitable src encoding nucleic acids are readily apparent to one skilled in the art.

### 2. Expression Vectors

A recombinant DNA molecule (rDNA) containing a DNA segment encoding a Src protein can be produced as described herein. In particular, an expressible rDNA can be produced by operatively (in frame, expressibly) linking a vector to a src encoding DNA segment. Thus, a recombinant DNA molecule is a hybrid DNA molecule comprising at least two nucleic acids of a nucleotide sequences not normally found together in nature.

The choice of vector to which the DNA segment is operatively linked depends directly, as is well known in the art, on the functional properties desired, e.g., protein expression, and the host cell to be transformed. A vector suitable for use in practicing the present invention is at least capable of directing the replication, and preferably also expression, of a structural gene included in the vector DNA segments to which it is operatively linked.

Both prokaryotic and eukaryotic expression vectors are familiar to one of ordinary skill in the art of vector construction, and are described by Ausubel, et

al., in Current Protocols in Molecular Biology, Wiley and Sons, New York (1993) and by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, (1989). These references also describe many of the general recombinant DNA methods referred to herein.

5 In one embodiment, a suitable vector includes a procaryotic replicon, i.e., a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extrachromosomally in a procaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition, those embodiments that include a procaryotic  
10 replicon also include a gene whose expression confers drug resistance to a bacterial host transformed therewith. Typical bacterial drug resistance genes are those that confer resistance to ampicillin or tetracycline.

Those vectors that include a procaryotic replicon can also include a procaryotic promoter capable of directing the expression (transcription and  
15 translation) of a structural gene in a bacterial host cell, such as E. coli, transformed therewith. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA  
20 segment of the present invention. Typical of such vector plasmids are pUC8, pUC9, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA), pRSET available from Invitrogen (San Diego, CA) and pPL and pKK223 available from Pharmacia, Piscataway, N.J.

Expression vectors compatible with eukaryotic cells, preferably those  
25 compatible with vertebrate cells, can also be used to form the recombinant DNA molecules of the present invention. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are pSVL and pKSV-10  
30 (Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC, #31255), pRc/CMV (Invitrogen, Inc.), the preferred vector described in the Examples, and the like eukaryotic expression vectors.

A particularly preferred system for gene expression in the context of this invention includes a gene delivery component, that is, the ability to deliver the gene to the tissue of interest. Suitable vectors are "infectious" vectors such as recombinant DNA viruses, adenovirus or retrovirus vectors which are engineered to express the desired protein and have features which allow infection of preselected target tissues. Particularly preferred is the replication competent avian sarcoma virus (RCAS) described herein.

Mammalian cell systems that utilize recombinant viruses or viral elements to direct expression may be engineered. For example, when using adenovirus expression vectors, the coding sequence of a polypeptide may be ligated to an adenovirus transcription/ translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted into the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the polypeptide in infected hosts (e.g., see Logan et al., Proc. Natl. Acad. Sci., USA, 81:3655-3659 (1984)). Alternatively, the vaccinia virus 7.5K promoter may be used (e.g., see, Mackett et al., Proc. Natl. Acad. Sci., USA, 79:7415-7419 (1982); Mackett et al., J. Virol., 49:857-864 (1984); Panicali et al., Proc. Natl. Acad. Sci., USA, 79:4927-4931 (1982)). Of particular interest are vectors based on bovine papilloma virus which have the ability to replicate as extrachromosomal elements (Sarver et al., Mol. Cell. Biol., 1:486 (1981)). Shortly after entry of this DNA into target cells, the plasmid replicates to about 100 to 200 copies per cell. Transcription of the inserted cDNA does not require integration of the plasmid into the host's chromosome, thereby yielding a high level of expression. These vectors can be used for stable expression by including a selectable marker in the plasmid, such as the neo gene. Alternatively, the retroviral genome can be modified for use as a vector capable of introducing and directing the expression of the polypeptide-encoding nucleotide sequence in host cells (Cone et al., Proc. Natl. Acad. Sci., USA, 81:6349-6353 (1984)). High level expression may also be achieved using inducible promoters, including, but not limited to, the metallothionine IIA promoter and heat shock promoters.

Recently, long-term survival of cytomegalovirus (CMV) promoter versus Rous sarcoma virus (RSV) promotor-driven thymidine kinase (TK) gene therapy in nude mice bearing human ovarian cancer has been studied. Cell killing efficacy of adenovirus-mediated CMV promoter-driven herpes simplex virus TK gene therapy was found to be 2 to 10 time more effective than RSV driven therapy. (Tong et al., 1999, Hybridoma 18(1):93-97). The design of chimeric promoters for gene therapy applications, which call for low level expression followed by inducible high-level expression has also been described. (Suzuki et al., 1996, Human Gene Therapy 7:1883-1893).

For long-term, high-yield production of recombinant proteins, stable expression is preferred. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with a cDNA controlled by appropriate expression control elements (e.g., promoter and enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. As mentioned above, the selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines.

For example, following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., Cell, 11:223 (1977)), hypoxanthine-guanine phosphoribosyltransferase (Szybalska et al, Proc. Natl. Acad. Sci., USA, 48:2026 (1962)), and adenine phosphoribosyltransferase (Lowy et al., Cell, 22:817 (1980)) genes, which can be employed in tk-, hgprt- or aprt- cells respectively. Also, antimetabolite resistance-conferring genes can be used as the basis of selection; for example, the genes for dhfr, which confers resistance to methotrexate (Wigler et al., Proc. Natl. Acad. Sci., USA, 77:3567 (1980); O'Hare et al., Proc. Natl. Acad. Sci., USA, 78:1527 (1981); gpt, which confers resistance to mycophenolic acid (Mulligan et al, Proc. Natl. Acad. Sci., USA, 78:2072, (1981)); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al, J. Mol. Biol., 150:1 (1981)); and hygromycin (Santerre et al, Gene,

30:147 (1984)). Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman et al, Proc. Natl. Acad. Sci., USA, 85:804 (1988)); and ODC (ornithine decarboxylase) which  
5 confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed., (1987)).

The principal vectors contemplated for human gene therapy are derived from retroviral origin. (Wilson, 1997, Clin. Exp. Immunol. 107(Sup. 1):31-32; Bank et al., 1996, Bioessays 18(12):999-1007; Robbins et al., 1998, Pharmacol. Ther. 80(1):35-47). The therapeutic potential of gene transfer and antisense therapy has stimulated the development of many vector systems for treating a variety of tissues. (vasculature, Stephan et al., 1997, Fundam. Clin. Pharmacol. 11(2):97-  
15 110; Feldman et al., 1997, Cardiovasc. Res. 35(3):391-404; Vassalli et al., 1997, Cardiovasc. Res. 35(3):459-69; Baek et al., 1998, Circ. Res. 82(3):295-305; kidney, Lien et al., 1997, Kidney Int. Suppl. 61:S85-8; liver, Ferry et al., 1998, Hum Gene Ther. 9(14):1975-81; muscle, Marshall et al., 1998, Curr. Opin. Genet. Dev. 8(3):360-5). In addition to these tissues, a critical target for human gene  
20 therapy is cancer, either the tumor itself, or associated tissues. (Runnebaum, 1997, Anticancer Res. 17(4B):2887-90; Spear et al., 1998, J. Neurovirol. 4(2):133-47).

Specific examples of viral gene therapy vector systems readily adaptable for use in the methods of the present invention are briefly described below. Retroviral gene delivery has been recently reviewed by Federspiel and Hughes (1998, Methods in Cell Biol. 52:179-214) which describes in particular, the avian  
25 leukemia virus (ALV) retrovirus family (Federspiel et al., Proc. Natl. Acad. Sci., USA, 93:4931 (1996); Federspiel et al., Proc. Natl. Acad. Sci., USA, 91:11241 (1994)). Retroviral vectors, including ALV and murine leukemia virus (MLV) are further described by Svoboda (1998, Gene 206:153-163).

30 Modified retroviral/adenoviral expression systems can be readily adapted for practice of the methods of the present invention. For example, murine leukemia virus (MLV) systems are reviewed by Karavanas et al., 1998, Crit. Rev. in Oncology/Hematology 28:7-30. Adenovirus expression systems are reviewed by

Von Seggern and Nemerow in Gene Expression Systems (ed. Fernandez & Hoeffler, Academic Press, San Diego, CA, 1999, chapter 5, pages 112-157).

- Protein expression systems have been demonstrated to have effective use both in vivo and in vitro. For example, efficient gene transfer to human squamous cell carcinomas by a herpes simplex virus (HSV) type 1 amplicon vector has been described. (Carew et al., 1998, Am. J. Surg. 176:404-408). Herpes simplex virus has been used for gene transfer to the nervous system. (Goins et al., 1997, J. Neurovirol. 3 (Sup. 1):S80-8). Targeted suicide vectors using HSV-TK has been tested on solid tumors. (Smiley et al., 1997, Hum. Gene Ther. 8(8):965-77).
- Herpes simplex virus type 1 vector has been used for cancer gene therapy on colon carcinoma cells. (Yoon et al., 1998, Ann. Surg. 228(3):366-74). Hybrid vectors have been developed to extend the length of time of transfection, including HSV/AAV (adeno-associated virus) hybrids for treating hepatocytes. (Fraefel et al., 1997, Mol. Med. 3(12):813-825).
- Vaccinia virus has been developed for human gene therapy because of its large genome. (Peplinski et al., 1998, Surg. Oncol. Clin. N. Am. 7(3):575-88). Thymidine kinase-deleted vaccinia virus expressing purine nucleoside pyrophosphorylase has been described for use as a tumor directed gene therapy vector. (Puhlman et al., 1999, Human Gene Therapy 10:649-657).
- Adeno-associated virus 2 (AAV) has been described for use in human gene therapy, however AAV requires a helper virus (such as adenovirus or herpes virus) for optimal replication and packaging in mammalian cells. (Snoeck et al., 1997, Exp. Nephrol. 5(6):514-20; Rabinowitz et al., 1998, Curr. Opin. Biotechnol. 9(5):470-5). However, in vitro packaging of an infectious recombinant AAV has been described, making this system much more promising. (Ding et al., 1997, Gene Therapy 4:1167-1172). It has been shown that the AAV mediated transfer of ecotropic retrovirus receptor cDNA allows ecotropic retroviral transduction of established and primary human cells. (Qing et al., 1997, J. Virology 71(7):5663-5667). Cancer gene therapy using an AAV vector expressing human wild-type p53 has been demonstrated. (Qazilbash et al., 1997, Gene Therapy 4:675-682). Gene transfer into vascular cells using AAV vectors has also been shown. (Maeda et al., 1997, Cardiovascular Res. 35:514-521). AAV has been demonstrated as a suitable vector for liver directed gene therapy. (Xiao et al., 1998, J. Virol. 72(12):10222-



6). AAV vectors have been demonstrated for use in gene therapy of brain tissues and the central nervous system. (Chamberlin et al., 1998, Brain Res. 793(1-2):169-75; During et al., 1998, Gene Therapy 5(6):820-7). AAV vectors have also been compared with adenovirus vectors (AdV) for gene therapy of the lung and transfer to human cystic fibrosis epithelial cells. (Teramoto et al., 1998, J. Virol. 72(11):8904-12).

Chimeric AdV/retroviral gene therapy vector systems which incorporate the useful qualities of each virus to create a nonintegrative AdV that is rendered functionally integrative via the intermediate generation of a retroviral producer cell. (Feng et al., 1997, Nat. Biotechnology 15(9):866-70; Bilbao et al., 1997, FASEB J 11(8):624-34). This powerful new generation of gene therapy vector has been adapted for targeted cancer gene therapy. (Bilbao et al., 1998, Adv. Exp. Med. Biol. 451:365-74). Single injection of AdV expressing p53 inhibited growth of subcutaneous tumor nodules of human prostate cancer cells. (Asgari et al., 1997, Int. J. Cancer 71(3):377-82). AdV mediated gene transfer of wild-type p53 in patients with advanced non-small cell lung cancer has been described. (Schuler et al., 1998, Human Gene Therapy 9:2075-2082). This same cancer has been the subject of p53 gene replacement therapy mediated by AdV vectors. (Roth et al., 1998, Semin. Oncol. 25(3 Suppl 8):33-7). AdV mediated gene transfer of p53 inhibits endothelial cell differentiation and angiogenesis in vivo. (Riccioni et al., 1998, Gene Ther. 5(6):747-54). Adenovirus-mediated expression of melanoma antigen gp75 as immunotherapy for metastatic melanoma has also been described. (Hirschowitz et al., 1998, Gene Therapy 5:975-983). AdV facilitates infection of human cells with ecotropic retrovirus and increases efficiency of retroviral infection. (Scott-Taylor, et al., 1998, Gene Ther. 5(5):621-9). AdV vectors have been used for gene transfer to vascular smooth muscle cells (Li et al., 1997, Chin. Med. J.(Engl) 110(12):950-4), squamous cell carcinoma cells (Goebel et al., 1998, Otolaryngol Head Neck Surg 119(4):331-6), esophageal cancer cells (Senmaru et al., 1998, Int J. Cancer 78(3):366-71), mesangial cells (Nahman et al., 1998, J. Investig. Med. 46(5):204-9), glial cells (Chen et al., 1998, Cancer Res. 58(16):3504-7), and to the joints of animals (Ikeda et al., 1998, J. Rheumatol. 25(9):1666-73). More recently, catheter-based pericardial gene transfer mediated by AcV vectors has been demonstrated. (March et al., 1999, Clin. Cardiol. 22(1

Suppl 1):123-9). Manipulation of the AdV system with the proper controlling genetic elements allows for the AdV-mediated regulable target gene expression in vivo. (Burcin et al., 1999, PNAS (USA) 96(2):355-60).

5 Alphavirus vectors have been developed for human gene therapy applications, with packaging cell lines suitable for transformation with expression cassettes suitable for use with Sindbis virus and Semliki Forest virus-derived vectors. (Polo et al., 1999, Proc. Natl. Acad. Sci., USA, 96:4598-4603). Noncytopathic flavivirus replicon RNA-based systems have also been developed. (Varnavski et al., 1999, Virology 255(2):366-75). Suicide HSV-TK gene containing sinbis virus  
10 vectors have been used for cell-specific targeting into tumor cells. (Iijima et al., 1998, Int. J. Cancer 80(1):110-8).

Retroviral vectors based on human foamy virus (HFV) also show promise as gene therapy vectors. (Trobridge et al., 1998, Human Gene Therapy 9:2517-2525). Foamy virus vectors have been designed for suicide gene therapy. (Nestler  
15 et al., 1997, Gene Ther. 4(11):1270-7). Recombinant murine cytomegalovirus and promoter systems have also been used as vectors for high level expression. (Manning et al., 1998, J. Virol. Meth. 73(1):31-9; Tong et al., 1998, Hybridoma 18(1):93-7).

Gene delivery into non-dividing cells has been made feasible by the generation  
20 of Sendai virus based vectors. (Nakanishi et al., 1998, J. Controlled Release 54(1):61-8).

In other efforts to enable the transformation of non-dividing somatic cells, lentiviral vectors have been explored. Gene therapy of cystic fibrosis using a replication-defective human immunodeficiency virus (HIV) based vector has been  
25 described. (Goldman et al., 1997, Human Gene Therapy 8:2261-2268). Sustained expression of genes delivered into liver and muscle by lentiviral vectors has also been shown. (Kafri et al., 1997, Nat. Genet. 17(3):314-7). However, safety concerns are predominant, and improved vector development is proceeding rapidly. (Kim et al., 1998, J. Virol. 72(2):994-1004). Examination of the HIV  
30 LTR and Tat yield important information about the organization of the genome for developing vectors. (Sadaie et al., 1998, J. Med. Virol. 54(2):118-28). Thus the genetic requirements for an effective HIV based vector are now better understood. (Gasmi et al., 1999, J. Virol. 73(3):1828-34). Self inactivating vectors, or

conditional packaging cell lines have been described. (for example Zuffery et al., 1998, J. Virol. 72(12):9873-80; Miyoshi et al., 1998, J. Virol. 72(10):8150-7; Dull et al., 1998, J. Virol. 72(11):8463-71; and Kaul et al., 1998, Virology 249(1):167-74). Efficient transduction of human lymphocytes and CD34+ cells by  
5 HIV vectors has been shown. (Douglas et al., 1999, Hum. Gene Ther. 10(6):935-45; Miyoshi et al., 1999, Science 283(5402):682-6). Efficient transduction of nondividing human cells by feline immunodeficiency virus (FIV) lentiviral vectors has been described, which minimizes safety concerns with using HIV based vectors. (Poeschla et al., 1998, Nature Medicine 4(3):354-357). Productive  
10 infection of human blood mononuclear cells by FIV vectors has been shown. (Johnston et al., 1999, J. Virol. 73(3):2491-8).

While many viral vectors are difficult to handle, and capacity for inserted DNA limited, these limitations and disadvantages have been addressed. For example, in addition to simplified viral packaging cell lines, Mini-viral vectors,  
15 derived from human herpes virus, herpes simplex virus type 1 (HSV-1), and Epstein-Barr virus (EBV), have been developed to simplify manipulation of genetic material and generation of viral vectors. (Wang et al., 1996, J. Virology 70(12):8422-8430). Adaptor plasmids have been previously shown to simplify insertion of foreign DNA into helper-independent Retroviral vectors. (1987, J.  
20 Virology 61(10):3004-3012).

Viral vectors are not the only means for effecting gene therapy, as several non-viral vectors have also been described. A targeted non-viral gene delivery vector based on the use of Epidermal Growth Factor/DNA polyplex (EGF/DNA) has been shown to result in efficient and specific gene delivery. (Cristiano, 1998,  
25 Anticancer Res. 18:3241-3246). Gene therapy of the vasculature and CNS have been demonstrated using cationic liposomes. (Yang et al., 1997, J. Neurotrauma 14(5):281-97). Transient gene therapy of pancreatitis has also been accomplished using cationic liposomes. (Denham et al., 1998, Ann. Surg. 227(6):812-20). A  
chitosan-based vector/DNA complexes for gene delivery have been shown to be  
30 effective. (Erbacher et al., 1998, Pharm. Res. 15(9):1332-9). A non-viral DNA delivery vector based on a terplex system has been described. (Kim et al., 1998, 53(1-3):175-82). Virus particle coated liposome complexes have also been used to

effect gene transfer. (Hirai et al., 1997, Biochem. Biophys. Res. Commun. 241(1):112-8).

Cancer gene therapy by direct tumor injections of nonviral T7 vector encoding a thymidine kinase gene has been demonstrated. (Chen et al., 1998, Human Gene Therapy 9:729-736). Plasmid DNA preparation is important for direct injection gene transfer. (Horn et al., 1995, Hum. Gene Ther. 6(5):656-73). Modified plasmid vectors have been adapted specifically for direct injection. (Hartikka et al., 1996, Hum. Gene Ther. 7(10):1205-17).

Thus, a wide variety of gene transfer/gene therapy vectors and constructs are known in the art. These vectors are readily adapted for use in the methods of the present invention. By the appropriate manipulation using recombinant DNA/molecular biology techniques to insert an operatively linked src (either active or inactive) into the selected expression/delivery vector, many equivalent vectors for the practice of the present invention can be generated.

#### 15 E. Methods For Modulation of Angiogenesis

The invention provides for a method for the modulation of angiogenesis in a tissue associated with a disease process or condition, and thereby effect events in the tissue which depend upon angiogenesis. Generally, the method comprises administering to the tissue associated with a disease process or condition, a composition comprising an angiogenesis-modulating amount of a Src protein or nucleic acid vector expressing active or inactive Src.

As described herein, any of a variety of tissues, or organs comprised of organized tissues, can support angiogenesis in disease conditions including skin, muscle, gut, connective tissue, joints, bones and the like tissue in which blood vessels can invade upon angiogenic stimuli.

The patient treated according to the present invention in its many embodiments is desirably a human patient, although it is to be understood that the principles of the invention indicate that the invention is effective with respect to all mammals, which are intended to be included in the term "patient". In this context, a mammal is understood to include any mammalian species in which treatment of tissue associated with diseases involving angiogenesis is desirable, particularly agricultural and domestic mammalian species.

Thus the method comprises administering to a patient a therapeutically effective amount of a physiologically tolerable composition containing a Src protein or DNA vector for expressing a Src protein in practicing the methods of the invention.

5       The dosage ranges for the administration of a Src protein depend upon the form of the protein, and its potency, as described further herein. The dosage amounts are large enough to produce the desired effect in which angiogenesis and the disease symptoms mediated by angiogenesis are ameliorated. The dosage should not be so large as to cause adverse side effects, however, such as  
10   hyperviscosity syndromes, pulmonary edema, congestive heart failure, and the like. Generally, the dosage will vary with the age, condition, sex of the patient, and extent of the disease in the patient, and can be readily determined by one of skill in the art. The dosage can also be adjusted by the individual physician in the event of any complication.

15       A therapeutically effective amount is an amount of Src protein, or nucleic acid encoding for (active or inactive) src protein, sufficient to produce a detectable modulation of angiogenesis in the tissue being treated, ie., an angiogenesis-modulating amount. Modulation of angiogenesis can be measured by CAM assay as described herein, or by other methods known to one skilled in the art.

20       The Src protein or nucleic acid vector expressing the Src protein can be administered parenterally by injection or by gradual infusion over time. Although the tissue to be treated can typically be accessed in the body by systemic administration, and therefore is most often treated by intravenous administration of therapeutic compositions, other tissues and delivery means are contemplated as  
25   well. Thus, compositions of the invention can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, transdermally, and can be delivered by peristaltic means.

      The therapeutic compositions containing a Src protein or nucleic acid vector expressing the Src protein can be conventionally administered intravenously, as by  
30   injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a

predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

In one preferred embodiment the reagent is administered in a single dosage intravenously. Localized administration can be accomplished by direct injection or  
5 by taking advantage of anatomically isolated compartments, isolating the microcirculation of target organ systems, reperfusion in a circulating system, or catheter based temporary occlusion of target regions of vasculature associated with diseased tissues.

The compositions are administered in a manner compatible with the dosage  
10 formulation, and in a therapeutically effective amount. The quantity to be administered and timing depends on the subject to be treated, capacity of the subject's system to utilize the active ingredient, and degree of therapeutic effect desired. Precise amounts of active ingredient required to be administered depend  
15 on the judgement of the practitioner and are peculiar to each individual. However, suitable dosage ranges for systemic application are disclosed herein and depend on the route of administration. Suitable regimes for administration are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations  
20 in the blood in the ranges specified for in vivo therapies are contemplated.

#### 1. Inhibition of Angiogenesis

Inhibition of angiogenesis is important in a variety of diseases, referred to as angiogenic diseases. Such diseases include, but are not limited to, inflammatory disorders such as immune and non-immune inflammation, chronic articular  
25 rheumatism and psoriasis, disorders associated with inappropriate or inopportune invasion of vessels such as diabetic retinopathy, neovascular glaucoma, restenosis, capillary proliferation in atherosclerotic plaques and osteoporosis, and cancer associated disorders, such as solid tumors, solid tumor metastases, angiofibromas, retrolental fibroplasia, hemangiomas, Kaposi sarcoma and the like cancers which  
30 require neovascularization to support tumor growth.

Thus, methods which inhibit angiogenesis in a tissue associated with a disease condition ameliorates symptoms of the disease and, depending upon the disease, can contribute to cure of the disease. In one embodiment, the invention

contemplates inhibition of angiogenesis, per se, in a tissue associated with a disease condition. The extent of angiogenesis in a tissue, and therefore the extent of inhibition achieved by the present methods, can be evaluated by a variety of methods.

5        Thus, in one related embodiment, a tissue to be treated is an inflamed tissue and the angiogenesis to be inhibited is inflamed tissue angiogenesis where there is neovascularization of inflamed tissue. In this class the method contemplates inhibition of angiogenesis in arthritic tissues, such as in a patient with chronic articular rheumatism, in immune or non-immune inflamed tissues, in psoriatic  
10       tissue and the like.

      In another related embodiment, a tissue to be treated is a retinal tissue of a patient with a retinal disease such as diabetic retinopathy, macular degeneration or neovascular glaucoma and the angiogenesis to be inhibited is retinal tissue angiogenesis where there is neovascularization of retinal tissue.

15       In an additional related embodiment, a tissue to be treated is a tumor tissue of a patient with a solid tumor, a metastases, a skin cancer, a breast cancer, a hemangioma or angiofibroma and the like cancer, and the angiogenesis to be inhibited is tumor tissue angiogenesis where there is neovascularization of a tumor tissue. Typical solid tumor tissues treatable by the present methods include lung,  
20       pancreas, breast, colon, laryngeal, ovarian, and the like tissues. Inhibition of tumor tissue angiogenesis is a particularly preferred embodiment because of the important role neovascularization plays in tumor growth. In the absence of neovascularization of tumor tissue, the tumor tissue does not obtain the required nutrients, slows in growth, ceases additional growth, regresses and ultimately  
25       becomes necrotic resulting in killing of the tumor.

      Stated in other words, the present invention provides for a method of inhibiting tumor neovascularization by inhibiting tumor angiogenesis according to the present methods. Similarly, the invention provides a method of inhibiting tumor growth by practicing the angiogenesis-inhibiting methods.

30       The methods are also particularly effective against the formation of metastases because (1) their formation requires vascularization of a primary tumor so that the metastatic cancer cells can exit the primary tumor and (2) their establishment in a secondary site requires neovascularization to support growth of the metastases.

In a related embodiment, the invention contemplates the practice of the method in conjunction with other therapies such as conventional chemotherapy directed against solid tumors and for control of establishment of metastases. The administration of angiogenesis inhibitor is typically conducted during or after  
5 chemotherapy, although it is preferably to inhibit angiogenesis after a regimen of chemotherapy at times where the tumor tissue will be responding to the toxic assault by inducing angiogenesis to recover by the provision of a blood supply and nutrients to the tumor tissue. In addition, it is preferred to administer the angiogenesis inhibition methods after surgery where solid tumors have been  
10 removed as a prophylaxis against metastases.

Insofar as the present methods apply to inhibition of tumor neovascularization, the methods can also apply to inhibition of tumor tissue growth, to inhibition of tumor metastases formation, and to regression of established tumors.

Restenosis is a process of smooth muscle cell (SMC) migration and  
15 proliferation into the tissue at the site of percutaneous transluminal coronary angioplasty which hampers the success of angioplasty. The migration and proliferation of SMC's during restenosis can be considered a process of angiogenesis which is inhibited by the present methods. Therefore, the invention also contemplates inhibition of restenosis by inhibiting angiogenesis according to  
20 the present methods in a patient following angioplasty procedures. For inhibition of restenosis, the inactivated tyrosine kinase is typically administered after the angioplasty procedure because the coronary vessel wall is at risk of restenosis, typically for from about 2 to about 28 days, and more typically for about the first 14 days following the procedure.

25 The present method for inhibiting angiogenesis in a tissue associated with a disease condition, and therefore for also practicing the methods for treatment of angiogenesis-related diseases, comprises contacting a tissue in which angiogenesis is occurring, or is at risk for occurring, with a composition comprising a therapeutically effective amount of an inactivated Src protein or vector expressing  
30 the protein.

Inhibition of angiogenesis and tumor regression occurs as early as 7 days after the initial contacting with the therapeutic composition. Additional or prolonged



exposure to inactive Src protein is preferable for 7 days to 6 weeks, preferably about 14 to 28 days.

2. Potential of Angiogenesis

In cases where it is desirable to promote or potentiate angiogenesis, administration of an active Src protein to the tissue is useful. The routes and timing of administration are comparable to the methods described hereinabove for inhibition.

F. Therapeutic Compositions

The present invention contemplates therapeutic compositions useful for practicing the therapeutic methods described herein. Therapeutic compositions of the present invention contain a physiologically tolerable carrier together with a Src protein or vector capable of expressing a Src protein as described herein, dissolved or dispersed therein as an active ingredient. In a preferred embodiment, the therapeutic composition is not immunogenic when administered to a mammal or human patient for therapeutic purposes.

As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a mammal without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like.

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art and need not be limited based on formulation. Typically such compositions are prepared as injectable either as liquid solutions or suspensions, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified or presented as a liposome composition.

The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of

auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient.

The therapeutic composition of the present invention can include pharmaceutically acceptable salts of any salt-forming components therein.

5 Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium,  
10 ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

Physiologically tolerable carriers for the active ingredients are well known in the art. Exemplary of liquid carriers are sterile aqueous solutions that contain no  
15 materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes.

20 Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary of such additional liquid phases are glycerin, vegetable oils such as cottonseed oil, and water-oil emulsions.

A therapeutic composition contains an angiogenesis-modulating amount of an Src protein of the present invention, or sufficient recombinant DNA expression  
25 vector to express an effective amount of Src protein, typically formulated to contain an amount of at least 0.1 weight percent of Src protein per weight of total therapeutic composition. A weight percent is a ratio by weight of Src protein to total composition. Thus, for example, 0.1 weight percent is 0.1 grams of Src protein per 100 grams of total composition. For DNA expression vectors, the  
30 amount administered depends on the properties of the expression vector, the tissue to be treated, and the like considerations.

### G. Article of Manufacture

The invention also contemplates an article of manufacture which is a labelled container for providing a Src protein of the invention. An article of manufacture comprises packaging material provided with appropriate labeling for the disease  
5 condition to be treated and a pharmaceutical agent contained within the packaging material.

The pharmaceutical agent in an article of manufacture is any of the compositions of the present invention suitable for providing a Src protein and formulated into a pharmaceutically acceptable form as described herein according  
10 to the disclosed indications. Thus, the composition can comprise a Src protein or a DNA molecule which is capable of expressing a Src protein. The article of manufacture contains an amount of pharmaceutical agent sufficient for use in treating a condition indicated herein, either in unit or multiple dosages.

The packaging material comprises a label which indicates the use of the  
15 pharmaceutical agent contained therein, e.g., for treating conditions assisted by the inhibition or potentiation of angiogenesis, and the like conditions disclosed herein. The label can further include instructions for use and related information as may be required for marketing. The packaging material can include container(s) for storage of the pharmaceutical agent.

20 As used herein, the term packaging material refers to a material such as glass, plastic, paper, foil, and the like capable of holding within fixed means a pharmaceutical agent. Thus, for example, the packaging material can be plastic or glass vials, laminated envelopes and the like containers used to contain a pharmaceutical composition including the pharmaceutical agent.

25 In preferred embodiments, the packaging material includes a label that is a tangible expression describing the contents of the article of manufacture and the use of the pharmaceutical agent contained therein.

### Examples

30 The following examples relating to this invention are illustrative and should not, of course, be construed as specifically limiting the invention. Moreover, such variations of the invention, now known or later developed, which would be within

the purview of one skilled in the art are to be considered to fall within the scope of the present invention hereinafter claimed.

1. Preparation of c-Src Expression Constructs

5 For preparing the expression constructs useful in modulating angiogenesis by the methods of the present invention, c-Src cDNA is manipulated and inserted into an expression construct/vector.

The cDNA sequence encoding for wild-type (i.e., endogenous) chicken c-Src is shown in FIG. 1 (SEQ ID NO.:2) with the encoded amino acid residue sequence shown in FIG. 2 (SEQ ID NO.:3). The encoded protein sequence is translated from the cDNA nucleotide positions 112 to 1713. The nucleic acid sequence corresponding to the nucleic acid sequence of human c-Src cDNA (SEQ ID NO.:4) and encoded amino acid residue (SEQ ID NO.:5) sequences are shown respectively in FIGs. 3 and 4. For the human protein sequence, the coding sequence begins at nucleotide position 134 to 1486 of the cDNA.

15 Wild-type as well as a number of mutated c-Src cDNAs were prepared. Mutated c-Src constructs were prepared by site-directed mutagenesis as described by Kaplan et al., EMBO J., 13:4745-4756 (1994). The mutated c-Src constructs for encoding mutated c-Src proteins for use in the methods of the present invention are described in Kaplan et al., id.. Kaplan et al. describe various mutated c-Src constructs and encoded proteins of useful for the practice of this invention. For example, Kaplan et al. depict several products of chicken c-src alleles in their FIG. 1, including SrcA and Src251.

25 Two categories of c-Src function to modulate angiogenesis are described. As previously discussed, one category contains Src molecules that increase angiogenesis and thus are considered to be active proteins. Wild-type Src along with various mutations are shown in the present invention to induce angiogenesis. One preferred mutation of wild type c-src which functions in this context with respect to its ability to induce blood vessel growth and therefore increase tumor weight in vivo is the Src A mutant having a point mutation at amino acid (aa) residue position 527 changing tyrosine 527 to phenylalanine. This site is normally a site for negative regulation by the c-Src kinase, referred to as kinase CSK. When CSK phosphorylates aa527 in the wild-type src, the protein is inactivated.

However, in mutated Src A, the regulatory tyrosine converted to phenylalanine thus conferring upon the protein a constitutively (i.e., permanently) active protein not subject to inactivation by phosphorylation.

5 Mutations in src have also been shown to have the opposite modulatory effect on angiogenesis, inhibiting angiogenesis instead of stimulating it. Such mutations are referred to as inactive src mutations. Proteins having mutation that confer this inhibitory activity are also referred to as dominant negative Src proteins in that they inhibit neovascularization, including that resulting from endogenous activity of Src as well as enhanced Src activity resulting from growth factor stimulation.  
10 Thus certain mutations of wild type c-src of the present invention can also function as a dominant negative with respect to their ability to block blood vessel growth, and for example, therefore decrease tumor weight in vivo.

Such preferred inhibitory c-Src protein includes the Src 251 in which only the first 251 amino acids of Src are expressed. This construct lacks the entire kinase  
15 domain and is therefore referred to as "kinase dead" src protein. A second construct is the Src (K295M) mutation in which the lysine amino acid residue 295 is mutated into a methionine. This point mutation in the kinase domain prevents ATP binding and also blocks kinase-dependent Src functions related to vascular cell and tumor cell signaling and proliferation.

20 For example, for the mutation at residue 527, as long as the resultant mutated amino acid residue is not tyrosine, serine, or threonine, the present invention contemplates that the presence of an alternate amino acid at the desired position will result in a Src protein with a desired active, angiogenesis promoting modulatory activity.

25 With respect to the point mutations, any mutation resulting in the desired inhibitory or stimulatory activity is contemplated for use in this invention. Fusion protein constructs combining the desired src protein (mutation or fragment thereof) with expressed amino acid tags, antigenic epitopes, fluorescent protein, or other such protein or peptides are also contemplated, so long as the desired modulating  
30 effect of the src protein is intact.

**TABLE I**

<u>Src/Mutation</u>	<u>Src Function</u>	<u>Effect on Angiogenesis</u>
c-Src	+	active stimulates
SrcA (T527F)	+	active stimulates
5 Src527(point)	+	active stimulates
Src251	-	inactive inhibits
Src (truncate)	-	inactive inhibits
Src(K295M)	-	inactive inhibits
Src295 (point)	-	inactive inhibits

10

One preferred expression construct for use in the present invention is the RCASBP(A) construct (SEQ ID NO.:1). This expression vector is based on a series of replication competent avian sarcoma viruses with an enhanced Bryan polymerase (BP) for improved titre, and is specific for the A type envelope glycoprotein expressed on normal avian cells (Reviewed in Methods in Cell Biology, 52:179-214 (1997); see also, Hughes et al., 1987, *J. Virol.* 61:3004-3012; Fekete & Cepko, 1993, *Mol. Cellular Biol.* 13(4):2604-2613; Itoh et al., 1996, *Development* 122:291-300; and Stott et al., 1998, *BioTechniques* 24:660-666). The complete sequence of RCASBP(A) (SEQ ID NO.:1) is given in the attached sequence listing, and a restriction map of the construct is depicted as FIG. 10, referred to herein as RCAS.

20

The original Src 251 construct was subcloned by Dr. Pam Schwartzberg, at NIH in Dr. Harold Varmus' laboratory. Briefly, cloning of a src cDNA sequence for expression thereof was accomplished by inserting a linker containing Not I-BstB1-Not I restriction sites into a unique Not I site in the 5' end of Src 251. Src has a unique Cla I site at the 3' end. Digestion of Src 251 with BstB1 and Cla I generated a BstB1-ClaI fragment which was then ligated into the Cla I site on RCASBP(A). A BstB1 overhang allows for ligation with a Cla I overhang that will not be recut with Cla I. The src constructs suitable for use in practicing the present invention are readily obtained in the above vector by first digesting the RCAS vector containing Src 251 with Not I and Cla I (in a DAM+ background) to allow for insertion of a similarly digested Src cDNA. Therefore this initial RCASBP(A) construct containing Src 251 was further used to subclone all other

25

30

Src constructs as described above and in Kaplan et al. (1994, The EMBO J. 13(20):4745-4756), into RCASBP(A) via a Not I-Cla I fragment generated through the Src 251 construction. To produce the desired c-src mutations in the cDNA, standard site-directed mutagenesis procedures familiar to one of ordinary skill in the art were utilized. PCR primers designed to incorporate the desired mutations were also designed with restriction sites to facilitate subsequent cloning steps. Entire segments of Src encoding nucleic acid sequences are deleted from the nucleic acid constructs through PCR amplification techniques based on the known cDNA sequences of chicken, human and the like homologs of Src and subsequent formation of new constructs.

In one embodiment of the invention, the 3' PCR primer used to amplify src nucleic acids also encodes for an in-frame sequence. Use of this primer adds a 9E10-myc epitope tag to the carboxyl terminus of the subsequent Src construct.

The following amino acids were added after amino acid 251 of Src to generate vector constructs containing the 9E10-myc epitope tag: VDMEQKLI AEEDLN (SEQ ID NO.: 6). Two separate PCRs were carried out for each construct and similar results were obtained. All mutant constructs constructed by PCR were also sequenced by PCR to confirm predicted DNA sequence of clones. Wild-type and mutated Src cDNAs for use in the expression systems of the present invention are also available from Upstate Biotech Laboratories, Lake Placid, NY which sells avian as well as human src, and several kinase dead and activated mutated forms.

Alternative expression vectors for use in the expressing the Src proteins of the present invention also include adenoviral vectors as described in US Patent Numbers 4,797,368, 5,173,414, 5,436,146, 5,589,377, and 5,670,488.

Alternative methods for the delivery of the Src modulatory proteins include delivery of the Src cDNA with a non-viral vector system as described in US Patent Number 5,675,954 and delivery of the cDNA itself as naked DNA as described in US Patent Number 5,589,466. Delivery of constructs of this invention is also not limited to topical application of a viral vector as described in the CAM assay system below. For example, viral vector preparations are also injected intravenously for systemic delivery into the vascular bed. These vectors are also targetable to sites of increased neovascularization by localized injection of a tumor, as an example.

In vitro expressed proteins are also contemplated for delivery thereof following expression and purification of the selected Src protein by methods useful for delivery of proteins or polypeptides. One such method includes liposome delivery systems, such as described in US Patent Numbers 4,356,167, 5,580,575, 5,542,935 and 5,643,599. Other vector and protein delivery systems are well known to those of ordinary skill in the art for use in the expression and/or delivery of the Src proteins of the present invention.

2. Characterization of the Untreated Chick Chorioallantoic Membrane (CAM)

A. Preparation of the CAM

Angiogenesis can be induced on the chick chorioallantoic membrane (CAM) after normal embryonic angiogenesis has resulted in the formation of mature blood vessels. Angiogenesis has been shown to be induced in response to specific cytokines or tumor fragments as described by Leibovich et al., Nature, 329:630 (1987) and Ausprunk et al., Am. J. Pathol., 79:597 (1975). CAMs were prepared from chick embryos for subsequent induction of angiogenesis and inhibition thereof. Ten day old chick embryos were obtained from McIntyre Poultry (Lakeside, CA) and incubated at 37°C with 60% humidity. A small hole was made through the shell at the end of the egg directly over the air sac with the use of a small crafts drill (Dremel, Division of Emerson Electric Co. Racine WI). A second hole was drilled on the broad side of the egg in a region devoid of embryonic blood vessels determined previously by candling the egg. Negative pressure was applied to the original hole, which resulted in the CAM (chorioallantoic membrane) pulling away from the shell membrane and creating a false air sac over the CAM. A 1.0 centimeter (cm) x 1.0 cm square window was cut through the shell over the dropped CAM with the use of a small model grinding wheel (Dremel). The small window allowed direct access to the underlying CAM.

The resultant CAM preparation was then either used at 6 days of embryogenesis, a stage marked by active neovascularization, without additional treatment to the CAM reflecting the model used for evaluating effects on embryonic neovascularization or used at 10 days of embryogenesis where angiogenesis has subsided. The latter preparation was thus used in this invention



for inducing renewed angiogenesis in response to cytokine treatment or tumor contact as described below.

### 3. CAM Angiogenesis Assay

#### A. Angiogenesis Induced by Growth Factors

5 Angiogenesis has been shown to be induced by cytokines or growth factors.

Angiogenesis was induced by placing a 5 millimeter (mm) X 5 mm Whatman filter disk (Whatman Filter paper No.1) saturated with Hanks Balanced Salt Solution (HBSS, GIBCO, Grand Island, NY) or HBSS containing 2 micrograms/milliliter ( $\mu\text{g/ml}$ ) recombinant basic fibroblast growth factor (bFGF) or vascular endothelial cell growth factor (VEGF) (Genzyme, Cambridge, MA) on the CAM of either a 9 or 10 day chick embryo in a region devoid of blood vessels and the windows were latter sealed with tape. Other concentrations of growth factors are also effective at inducing blood vessel growth. For assays where inhibition of angiogenesis is evaluated with intravenous injections of antagonists, angiogenesis is first induced with 1-2  $\mu\text{g/ml}$  bFGF or VEGF in fibroblast growth medium. Angiogenesis was monitored by photomicroscopy after 72 hours.

#### B. Embryonic Angiogenesis

The CAM preparation for evaluating the effect of angiogenesis inhibitors on the natural formation of embryonic neovasculature is the 6 day embryonic chick embryo as previously described. At this stage in development, the blood vessels are undergoing de novo growth and thus provides a useful system for assessing angiogenesis modulation by the Src proteins of the present invention. The CAM system is prepared as described above with the exception that the assay is performed at embryonic day 6 rather than at day 9 or 10.

### 25 4. Modulation of Angiogenesis as Measured in the CAM Assay

To assess the effect of Src proteins on angiogenesis, the following assays were performed on 10 day old chick CAM preparations. Five  $\mu\text{g}$  of RCAS constructs prepared as described in Example 1 were transfected into the chicken immortalized fibroblast line, DF-1 (gift of Doug Foster, U. of Minn.). This cell line as well as primary chick embryo fibroblasts were capable of producing virus, however the DF-1 cell line produced higher titres. Viral supernatants were collected from subconfluent DF-1 producer cell lines in serum free CLM media [composition: F-10 media base supplemented with DMSO, folic acid, glutamic acid, and MEM

vitamin solution]. Thirty-five ml of viral supernatant were concentrated by ultracentrifugation at 4°C for 2 hours at 22,000 rpm. These concentrated viral pellets were resuspended in 1/100 the original volume in serum-free CLM media, aliquoted and stored at -80°C. The titre was assessed by serial dilution of a control viral vector having a nucleotide sequence encoding green fluorescent protein (GFP), referred to as RCAS-GFP, infection on primary chick embryo fibroblasts that were incubated for 48-72 hours. The titres of viral stock that were obtained following concentration routinely exceeded 10<sup>8</sup> I.u./ml. For the CAM assay using the viral stocks, cortisone acetate soaked Whatman filter disks 6 mm in diameter were prepared in 3 mg/ml cortisone acetate for 30 minutes in 95% ethanol. The disks were dried in a laminar flow hood and then soaked on 20 µl of viral stock per disk for 10 minutes. These disks were applied to the CAM of 9 or 10 day chick embryos and sealed with cellophane tape and incubated at 37°C for 18-24 hr. Then either mock PBS or growth factors were added at a concentration of 5 µg/ml to the CAM in a 20 µl volume of the appropriate virus stock as an additional boost of virus to the CAM tissue. After 72 hours, the CAMs were harvested and examined for changes in the angiogenic index as determined by double blind counting of the number of branch points in the CAM underlying the disk. For kinase assays, the tissue underlying the disk was harvested in RIPA, homogenized with a motorized grinder and Src immunoprecipitated from equivalent amounts of total protein and subjected to an in vitro kinase assay using a FAK-GST fusion protein as a substrate. For the immunofluorescence studies, CAM tissue underlying the disks were frozen in OCT, a cryopreservative, sectioned at 4 µm, fixed in acetone for 1 minute, incubated in 3% normal goat serum for 1 hour, followed by an incubation in primary rabbit anti-phosphorylated ERK antibody as described previously (Eliceiri et al., J. Cell Biol., 140:1255-1263 (1998), washed in PBS and detected with a fluorescent secondary antibody.

A. Activation of Endogenous Src by bFGF or VEGF

To assess the effects of growth factors on Src activity in modulating angiogenesis, the following assays were performed. Tissue extracts of 10 day old chick CAMs that had been exposed to bFGF or VEGF (2 µg/ml) for 2 hours were lysed. Endogenous Src was immunoprecipitated from equivalent amounts of total protein and subjected to an in vitro immune complex kinase assay using a FAK-

GST fusion protein as a substrate, electrophoresed and transferred to nitrocellulose.

The results of the assay are shown in FIG. 5 where the increase in Src activity is evident in the increased density of the gel with either bFGF or VEGF treatment as compared to untreated (mock) samples that are indicative of baseline Src activity in the CAM assay. Both bFGF and VEGF resulted in approximately a 2 fold increase of endogenous Src activity present in the CAM. The above kinase assay blot was also probed with an anti-Src antibody as a loading control for equivalent Src and IgG content.

10        B.     Effect of Retrovirus-Mediated Gene Expression of Src A on Angiogenesis in the Chick CAM

The following assay was performed to assess the effect of mutated Src proteins on angiogenesis in the CAM preparation. For the assay, 9 day old chick CAMs were exposed to RCAS-Src A or RCAS-GFP expressing retroviruses or buffer for 72 hour following the protocol described above.

The results of this assay are shown in FIG. 6A where the level of angiogenesis was quantified as described above. Representative photomicrographs (4x) were taken with a stereomicroscope as shown in FIG. 6B. Baseline endogenous Src activity has an angiogenic index of approximately 50. In contrast, CAMs treated with retroviral vector-expressed RCAS-Src A having a point mutation at amino acid residue position 527 from a tyrosine to a phenylalanine resulted in an enhancement (induction) of angiogenesis of an angiogenic index of approximately 90. The enhancement of Src-A mediated angiogenesis is also evident in the photographs shown in FIG. 6B.

25        C.     Retroviral Expression of Src A Activates Vascular MAP Kinase Phosphorylation

The effect of Src A as compared to growth factors VEGF and PMA on vascular MAP kinase phosphorylation was also assessed following the assay procedures described above and herein. Tissue extracts of 10 day old chick CAMs exposed to VEGF or PMA (another mitogen at a comparable concentration) for 30 minutes were compared to those infected with Src A-expressing retrovirus for 48 hours. Src was then immunoprecipitated from equivalent amounts of total protein extract and subjected to an in vitro immune complex kinase assay using a

FAK-GST fusion protein as a substrate, electrophoresed and transferred to nitrocellulose.

The results of this assay are shown in FIG. 7A where untreated CAMs (NT) exhibit base-line endogenous Src-mediated vascular MAP kinase phosphorylation. Both VEGF and PMA resulted in an approximate 2 fold increase over baseline. In contrast, Src A enhanced the activity approximately 5 to 10 fold over that seen with untreated samples.

Aliquots of the above whole tissue lysates were also measured for endogenous ERK phosphorylation by immunoblotting with an anti-phospho-ERK antibody as shown in FIG. 7B. For this assessment, 10 day old CAMs were infected with either mock RCAS or RCAS that expresses SRC A. After two days, CAMs were dissected, cryopreserved in OCT and sectioned at 4  $\mu$ m. Sections were immunostained with an anti-phosphorylated ERK antibody (New England Biolabs), washed and detected with a goat anti-rabbit FITC-conjugated secondary antibody. Fluorescent images were captured on a cooled-CCD camera (Princeton Inst.). The photomicrographs indicate enhanced immunofluorescence with Src A-treated preparations compared to mock controls.

D. Selective Requirement for Src Activity During VEGF, but Not bFGF-Induced Angiogenesis

To assess the effect of Src modulatory activity on growth factor induced angiogenesis, the following assays were performed. Nine day old chick CAMs were exposed to the retroviral vector preparation that expressed the dominant negative Src mutation referred to as Src 251 or Src K295M as previously described. RCAS-Src 251 or control RCAS-GFP retroviruses or buffer CAMS were treated for 20 hours and then incubated for an additional 72 hours in the presence or absence of bFGF or VEGF.

The level of angiogenesis, quantified as described above, is shown in FIG. 8A. Representative photomicrographs (6x), shown in FIG. 8B, were taken with a stereomicroscope. FIG. 8C illustrates a blot probed with an anti-Src antibody to confirm the expression of Src 251 in transfected cells as compared to mock treatments.

The results of the assays described above indicate that both bFGF and VEGF treated CAMS in the presence of RCAS-GFP controls induced angiogenesis over

the Src-mediated baseline angiogenesis seen with mock or untreated CAM preparations. The expressed dominant negative mutant Src 251 was effective at inhibiting VEGF-induced angiogenesis back to baseline levels while not effective at inhibiting bFGF-mediated angiogenesis. The photomicrographs shown in FIG. 8B pictorially confirm the data shown in FIG. 8A. Thus, retrovirally expressed Src 251 is an effective angiogenesis inhibitor, when angiogenesis is induced with VEGF.

Applications of the Src proteins of this invention with other angiogenesis models as described in the Examples below are contemplated in the present invention.

5     5. Regression of Tumor Tissue Growth With Src Modulators as Measured by In Vivo Rabbit Eye Model Assay

The effect of Src modulators on growth factor-induced angiogenesis can be observed in naturally transparent structures as exemplified by the cornea of the eye. New blood vessels grow from the rim of the cornea, which has a rich blood supply, toward the center of the cornea, which normally does not have a blood supply. Stimulators of angiogenesis, such as bFGF, when applied to the cornea induce the growth of new blood vessels from the rim of the cornea. Antagonists of angiogenesis, applied to the cornea, inhibit the growth of new blood vessels from the rim of the cornea. Thus, the cornea undergoes angiogenesis through an invasion of endothelial cells from the rim of the cornea into the tough collagen-packed corneal tissue which is easily visible. The rabbit eye model assay therefore provides an in vivo model for the direct observation of stimulation and inhibition of angiogenesis following the implantation of compounds directly into the cornea of the eye.

25     A.     In Vivo Rabbit Eye Model Assay

1)     Angiogenesis Induced by Growth Factors

Angiogenesis is induced in the in vivo rabbit eye model assay with growth factors bFGF or VEGF and is described in the following sections.

30     a.     Preparation of Hydron Pellets Containing Growth Factor and Monoclonal Antibodies

Hydron polymer pellets containing growth factor are prepared as described by D'Amato, et al., Proc. Natl. Acad. Sci., USA, 91:4082-

4085 (1994). The individual pellets contain 650 ng of the growth factors separately bound to sucralfate (Carafet, Marion Merrell Dow Corporation) to stabilize the growth factor and ensure its slow release into the surrounding tissue. In addition, hydron pellets are prepared containing a desired Src-expressing retrovirus as previously described. The pellets are cast in specially prepared Teflon pegs that have a 2.5 mm core drilled into their surfaces. Approximately 12 ul of casting material is placed into each peg and polymerized overnight in a sterile hood. Pellets are then sterilized by ultraviolet irradiation. Effects of Src proteins are then assessed as previously described.

6. In Vivo Regression of Tumor Tissue Growth With Src Modulators As Measured by Chimeric Mouse:Human Assay

An in vivo chimeric mouse:human model is generated by replacing a portion of skin from a SCID mouse with human neonatal foreskin. The in vivo chimeric mouse:human model is prepared essentially as described in Yan, et al., J. Clin. Invest., 91:986-996 (1993). Briefly, a 2 cm<sup>2</sup> square area of skin is surgically removed from a SCID mouse (6-8 weeks of age) and replaced with a human foreskin. The mouse is anesthetized and the hair removed from a 5 cm<sup>2</sup> area on each side of the lateral abdominal region by shaving. Two circular graft beds of 2 cm<sup>2</sup> are prepared by removing the full thickness of skin down to the fascia. Full thickness human skin grafts of the same size derived from human neonatal foreskin are placed onto the wound beds and sutured into place. The graft is covered with a Band-Aid which is sutured to the skin. Micropore cloth tape is also applied to cover the wound.

The M21-L human melanoma cell line or MDA 23.1 breast carcinoma cell line (ATCC HTB 26;  $\alpha_v\beta_3$  negative by immunoreactivity of tissue sections with mAb LM609), are used to form the solid human tumors on the human skin grafts on the SCID mice. A single cell suspension of  $5 \times 10^6$  M21-L or MDA 23.1 cells is injected intradermally into the human skin graft. The mice are then observed for 2 to 4 weeks to allow growth of measurable human tumors.

After a measurable tumor is established, retrovirus preparations of the present invention or PBS is injected into the mouse tail vein. Following a 2-3 week period, the tumor is excised and analyzed by weight and histology. The effect of expressed Src proteins of the present invention on the tumors is then assessed.

7. In Vitro Regression of Human Tumor Tissue Growth With Src Modulators As Measured by CAM Assay

Tumor growth depends on angiogenesis (Folkman, 1992; Weidner et al., 1991; Brooks et al., 1994b). In fact, recent reports suggest that tumor growth is susceptible to the anti-angiogenic effects of VEGF receptor antagonists (Kim et al., 1993). Therefore, we examined whether suppression of angiogenesis by delivery of kinase-deleted Src 251 would influence the growth of a human medulloblastoma (DAOY), a highly angiogenic tumor known to produce VEGF and very little bFGF (data not shown).

The 3 and 6 day DAOY medulloblastoma tumor growth assays were performed in the chick CAM essentially as previously described (Brooks et al., 1994).  $5 \times 10^6$  DAOY cells cultured in RPMI 1640 containing 10% fetal calf serum were washed and seeded on the CAM of a 10 day embryo to produce DAOY tumor fragments. After 7 days 50 mg tumor fragments were dissected and reseeded on another 10 day embryo and incubated for another 3 or 6 days with the topical application ( $25\mu\text{l}$ ) of either control RCAS-GFP retrovirus, RCAS-Src 251, or mock treatment. Using the whole tissue confocal imaging of infected tumors as a guide we were able to determine that there was significant expression of the RCAS constructs around and within the tumor fragment with this topical approach. Tumor resections and weighing were performed in a double blind manner removing only the easily definable solid tumor mass (Brooks et al., 1994). The wet tumor weights after 3 or 6 days were compared with initial weight and the percent change of tumor weight determined for each group.

These tumors readily grow on the CAM and produces active angiogenesis (FIG. 9) allowing us to selectively target the avian-derived tumor vasculature by using an avian-specific RCAS retrovirus.

FIG. 9 depicts results that show retroviral delivery of RCAS-Src 251 to human tumors growing on the chick CAM reverses tumor growth. FIG. 9A shows human medulloblastomas that were grown on the CAM of chick embryos as described above. Retrovirus containing RCAS-GFP or RCAS-Src 251 was topically applied to preestablished tumors of greater than 50 mg. A representative micrograph of a medulloblastoma tumor fragment infected with RCAS-GFP expressing GFP reveals exclusive expression in the tumor blood vessels

(arrowhead) as detected by optical sectioning with a Bio Rad laser confocal scanning microscope (bar=500 $\mu$ m). FIG. 9B shows results from tumors treated as above that were allowed to grow for 3 or 6 days after which they were resected and wet weights determined. Data are expressed as the mean change in tumor weight (from the 50 mg tumor starting weight) +/- SEM of 2 replicates. RCAS-Src 251 had a significant impact on tumor growth after 3 days (\*,  $P < 0.002$ ) and 6 days (\*\*,  $P < 0.05$ ). FIG. 9C shows representative stereomicrographs of medulloblastoma tumors surgically removed from the embryos were taken with an Olympus stereomicroscope (bar=350 $\mu$ m). (Lower panel) A high magnification micrograph of each tumor showing the vasculature of each tumor in detail (bar=350 $\mu$ m). The arrowhead indicates blood vessel disruption in RCAS-Src251-treated tumors.

The results show that delivery of RCAS containing Src 251 to preestablished medulloblastomas resulted in selective viral expression in the tumor-associated blood vessels (FIG. 9A) and this ultimately led to the regression of these tumors within the span of six days (FIG. 9B). Importantly, the tumor-associated blood vessels in animals treated with virus containing Src 251 were severely disrupted and fewer in number compared to the tumor vessels in control animals (FIG. 9C). The fact that RCAS-GFP infected tumors showed GFP localization only in the tumor vasculature suggests that the anti-tumor effects observed with retrovirally delivered Src 251 were due to its anti-angiogenic properties.

The foregoing examples and the accompanying description are illustrative, and are not to be taken as limiting. The present invention also is not to be limited in scope by the cell line deposited, since the deposited embodiment is intended as a single illustration of one aspect of the invention. Any cell line that is functionally equivalent is within the scope of this invention. The deposit of material does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustration that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.



## What Is Claimed Is:

1. An article of manufacture comprising packaging material and a pharmaceutical composition contained within said packaging material, wherein said pharmaceutical composition is capable of modulating angiogenesis in a tissue associated with a disease condition, wherein said packaging material comprises a label which indicates that said pharmaceutical composition can be used for treating disease conditions by modulating angiogenesis, and wherein said pharmaceutical composition comprises a Src protein or an oligonucleotide having a nucleotide sequence capable of expressing said protein.
2. The article of manufacture of claim 1 wherein said Src protein is an active Src protein and said modulating potentiates angiogenesis.
3. The article of manufacture of claim 2 wherein said active Src protein is Src A.
4. The article of manufacture of claim 2 wherein said tissue has poor circulation.
5. The article of manufacture of claim 1 wherein said tyrosine kinase Src protein is an inactive Src protein and said modulating inhibits angiogenesis.
6. The article of manufacture of claim 5 wherein said inactive Src protein is Src 251 or Src K295M.
7. The article of manufacture of claim 5 wherein said tissue is inflamed and said condition is arthritis or rheumatoid arthritis.
8. The article of manufacture of claim 5 wherein said tissue is a solid tumor or solid tumor metastasis.
9. The article of manufacture of claim 8 wherein said administering is conducted in conjunction with chemotherapy.
10. The article of manufacture of claim 5 wherein said tissue is retinal tissue and said condition is retinopathy, diabetic retinopathy or macular degeneration.
11. The article of manufacture of claim 5 wherein said tissue is at the site of coronary angioplasty and said condition is restenosis.
12. The article of manufacture of claim 1 wherein said administering comprises intravenous, transdermal, intrasynovial, intramuscular, or oral administration.

13. The article of manufacture of claim 1 wherein said administering comprises a single dose intravenously.

14. The article of manufacture of claim 1 wherein said pharmaceutical composition further comprises a liposome.

5 15. The article of manufacture of claim 1 wherein said pharmaceutical composition comprises a viral expression vector capable of expressing said nucleotide sequence.

10 16. The article of manufacture of claim 1 wherein said pharmaceutical composition comprises an non-viral expression vector capable of expressing said nucleotide sequence.

17. A method for modulating angiogenesis in a tissue associated with a disease condition comprising administering to said tissue a pharmaceutical composition comprising a Src protein or a nucleotide sequence capable of expressing said protein.

15 18. The method of claim 17 wherein said Src protein is an active Src protein and said modulating potentiates angiogenesis.

19. The method of claim 18 wherein said active Src protein is Src A.

20. The method of claim 18 wherein said tissue has poor circulation.

20 21. The method of claim 17 wherein said Src protein is an inactive Src protein and said modulating inhibits angiogenesis.

22. The method of claim 21 wherein said inactive Src protein is Src 251 or Src K295M.

23. The method of claim 21 wherein said tissue is inflamed and said condition is arthritis or rheumatoid arthritis.

25 24. The method of claim 21 wherein said tissue is a solid tumor or solid tumor metastasis.

25. The method of claim 24 wherein said administering is conducted in conjunction with chemotherapy.

30 26. The method of claim 21 wherein said tissue is retinal tissue and said condition is retinopathy, diabetic retinopathy or macular degeneration.

27. The method of claim 21 wherein said tissue is at the site of coronary angioplasty and said tissue is at risk for restenosis.

28. The method of claim 17 wherein said administering comprises intravenous, transdermal, intrasynovial, intramuscular, or oral administration.

29. The method of claim 17 wherein said administering comprises a single dose intravenously.

5 30. The method of claim 17 wherein said pharmaceutical composition further comprises a liposome.

31. The method of claim 17 wherein said pharmaceutical composition comprises an retroviral expression vector capable of expressing said nucleotide sequence.

10 32. The method of claim 17 wherein said pharmaceutical composition comprises an non-viral expression vector capable of expressing said nucleotide sequence.

33. A pharmaceutical composition for stimulating angiogenesis in a target mammalian tissue comprising a viral gene transfer vector containing a nucleic acid and pharmaceutically acceptable carrier or excipient; said nucleic acid having a nucleic acid segment encoding for a src protein, said src protein having any amino acid residue at codon 527 except for tyrosine, serine or threonine.

15 34. A pharmaceutical composition for stimulating angiogenesis in a target mammalian tissue comprising a non-viral gene transfer vector containing a nucleic acid and pharmaceutically acceptable carrier or excipient; said nucleic acid having a nucleic acid segment encoding for a src protein and said src protein having any amino acid residue at codon 527 except tyrosine, serine or threonine.

20 35. A pharmaceutical composition for inhibiting angiogenesis in a target mammalian tissue comprising a viral gene transfer vector containing a nucleic acid and pharmaceutically acceptable carrier or excipient; said nucleic acid having a nucleic acid segment encoding for a src protein having no kinase activity.

25 36. A pharmaceutical composition for inhibiting angiogenesis in a target mammalian tissue comprising a non-viral gene transfer vector containing a nucleic acid and pharmaceutically acceptable carrier or excipient; said nucleic acid having a nucleic acid segment encoding for a src protein, said src protein having no kinase activity.

30 37. A pharmaceutical composition for stimulating angiogenesis in a target mammalian tissue comprising a therapeutic amount of a src protein in a

pharmaceutically acceptable carrier or excipient; said src protein having any amino acid residue at codon 527 except tyrosine, serine or threonine.

38. A pharmaceutical composition for inhibiting angiogenesis in a target mammalian tissue comprising a a src protein in a pharmaceutically acceptable carrier or excipient; said src protein having no kinase activity.
- 5

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## CHICKEN c-SRC cDNA

(SEQ ID NO:2)

1 tctgacaccc atctgtctgt ctgtctgtgt gctgcaggag ctgagctgac tctgctgtgg  
61 cctcgcgtac cactgtggcc aggcggtagc tgggacgtgc agcccaccac catggggagc  
121 agcaagagca agcccaagga cccagccag cgccggcgca gcctggagcc acccgacagc  
181 acccaccacg ggggattccc agcctcgag accccaaca agacagcagc ccccgacacg  
241 caccgcaccc ccagccgctc ctttgggacc gtggccaccg agcccaagct cttcgggggc  
301 ttcaacattt ctgacaccgt tacgtcgccg cagcgtgccg gggcactggc tggcggcgtc  
361 accatttcg tggctctcta cgactacgag tcccggactg aaacggactt gtccttcaag  
421 aaaggagaac gcctgcagat tgtaacaac acggaagggtg actggtggct ggctcattcc  
481 ctactacag gacagacggg ctacatcccc agtaactatg tcgcgccctc agactccatc  
541 caggctgaag agtggtactt tgggaagatc actcgtcggg agtccgagcg gctgctgtc  
601 aaccccgaaa acccccgggg aaccttcttg gtccgggaga gcgagacgac aaaaggtgcc  
661 tattgcctct ccgtttctga cttgacaac gccaaggggc tcaatgtgaa gactacaag  
721 atccgcaagc tggacagcgg cggtcttac atcacctcac gcacacagtt cagcagcctg  
781 cagcagctgg tggcctacta ctcaaacat gctgatggct tgtgccaccg cctgaccaac  
841 gtctgcccc agtccaagcc ccagaccag ggactcgcca aggacgcgtg ggaaatcccc  
901 cgggagtcgc tgcggctgga ggtgaagctg gggcagggct gctttggaga ggtctggatg  
961 gggacctgga acggcaccac cagagtggcc ataaagactc tgaagcccgg caccatgtcc  
1021 ccggaggcct tcctgcagga agcccaagtg atgaagaagc tccggcatga gaagctggtt  
1081 cagctgtacg cagtgggtgc ggaagagccc atctacatc tactgagta catgagcaag  
1141 gggagcctcc tggatttct gaaggagag atgggcaagt acctgcggct gccacagctc  
1201 gtcgatatgg ctgctcagat tgcacccggc atggcctatg tggagaggat gaactacgtg  
1261 caccgagacc tgcgggcggc caacatcctg gtgggggaga acctggtgtg caaggtggct  
1321 gactttgggc tggcacgct catcgaggac aacgagtaca cagcacggca aggtgccaaag  
1381 ttcccatca agtggacagc ccccgaggca gccctctatg gccggttac catcaagtcg  
1441 gatgtctggt cttcggcat cctgctgact gagctgacca ccaagggccg ggtgccatac  
1501 ccagggatgg tcaacaggga ggtgctggac caggtggaga ggggctaccg catgccctgc  
1561 ccgcccagat gccccagtc gctgcatgac ctcatgtgcc agtgcctggc gagggacct  
1621 gaggagcggc ccactttga gtacctgcag gccttcctgg aggactactt cacctcgaca  
1681 gagccccagt accagcctgg agagaaccta taggcctgga gctcctcctg gaccagaggc  
1741 ctgcgtgtgg ggtacaggg

FIG. 1

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## CHICKEN cSRC ENCODED PROTEIN

(SEQ ID NO:3)

MGSSKSKPKDPSQRRRSLEPPDSTHHGGFPASQTPNKTA  
PDTHRTPSRSFGTVATEPKLFGGFNTSDTVTSPQRAGALA  
GGVTTFVALYDYESRTETDLSFKKGERLQIVNNTEGDWWL  
AHS�TTGQTGYIPSNYVAPSDSIQAEWYFGKITRRESER  
LLNPENPRGTFLVRESETTKGAYCLSVSDFDNAKGLNVK  
HYKIRKLDSGGFYITSRTQFSSLQQLVAYYSKHADGLCHR  
LTNVCPTSKPQTQGLAKDAWEIPRESLRLEVKLGQGCFIGE  
VWMGTWNGTTRVAIKTLKPGTMSPEAFLQEAQVMKKLRHE  
KLVQLYAVVSEPIYIVTEYMSKGSLLDFLKGEMGKYLRL  
PQLVDMAAQIASGMAYVERMNYVHRDLRAANILVGENL  
VCKVADFGLARLIEDNEYTARQGAKFPIKWTAPEAALYGR  
FTIKSDVWSFGILLTELTTKGRVPYPGMVNREVLDQVERG  
YRMPCPPECPESLHDLMCQCWRRDPEERPTFEYLQAFLE  
DYFTSTEPQYQPGENL

FIG. 2

3/10

## HUMAN c-SRC cDNA

(SEQ ID NO:4)

1 gcgccgcgtc ccgcaggccg tgatccgcc cgcgcgaggagg tggcccggac cgcaagtgc  
61 caagagagct ctaatggtac caagtgcag gttggcttta ctgtgactcg gggacgccag  
121 agtcctgag aagatgtcag caatacaggc cgcctggcca tccggtacag aatgtattgc  
181 caagtacaac ttccacggca ctgccgagca ggacctgccc ttctgcaaag gagacgtgct  
241 caccattgtg gccgtcacca aggaccccaa ctggtacaaa gccaaaaaca aggtgggccc  
301 tgagggcac atcccagcca actacgtcca gaagcgggag ggcgtgaagg cgggtaccaa  
361 actcagctc atgccttgg tccacggcaa gatcacagg gagcaggctg agcggcttct  
421 gtaccgccg gagacaggcc tgttcttgg gcgggagagc accaactacc ccggagacta  
481 cacgtgtgc gtgagctgcg acggcaaggt ggagcactac cgcacatgt accatgccag  
541 caagtcagc atcgacgagg aggtgtactt tgagaacctc atgcagctgg tggagcacta  
601 caccacagac gcagatggac tctgtacgc ctcattaaa ccaaaggta tggagggcac  
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FIG. 3

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## HUMAN c-SRC ENCODED PROTEIN

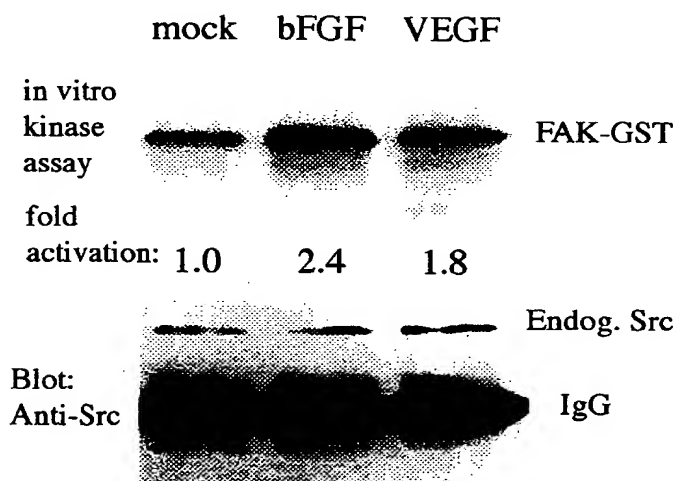
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**FIG. 4**

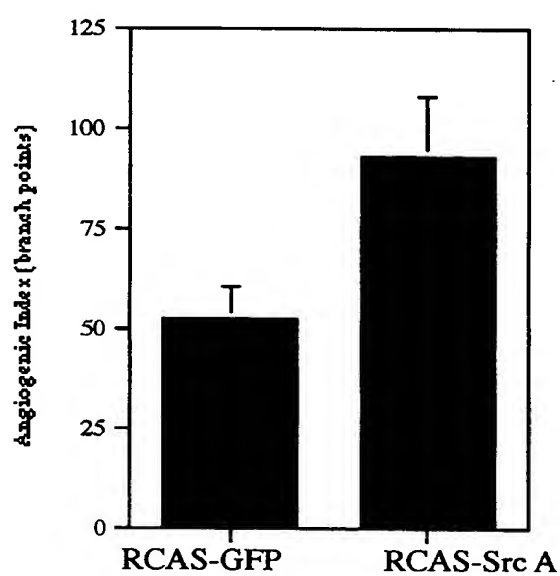


### Activation of endogenous Src activity by bFGF and VEGF

**FIG. 5**

Effect of RCAS-mediated expression of  
Src A on angiogenesis in the chick CAM

A.



B.

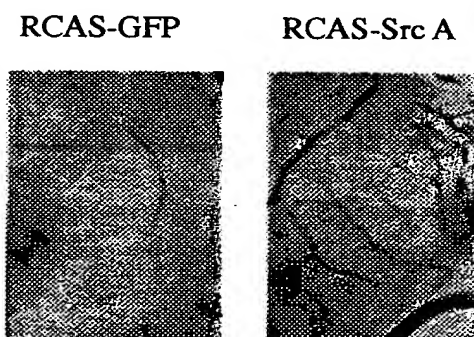
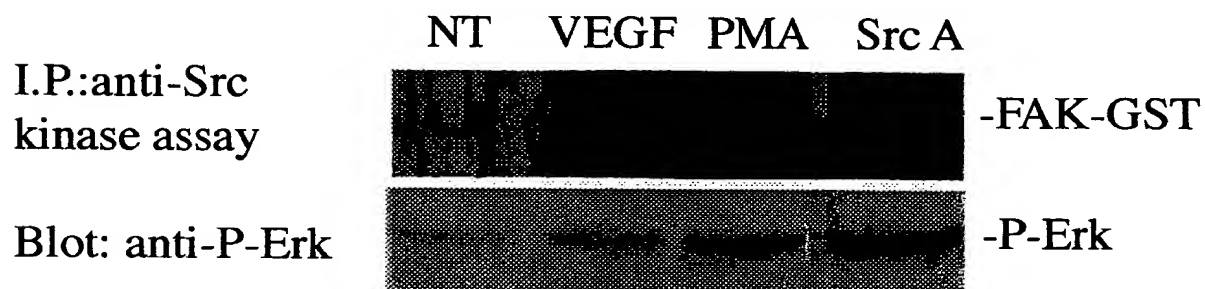


FIG. 6

## Retroviral expression of Src A activates vascular MAP kinase phosphorylation



Mock

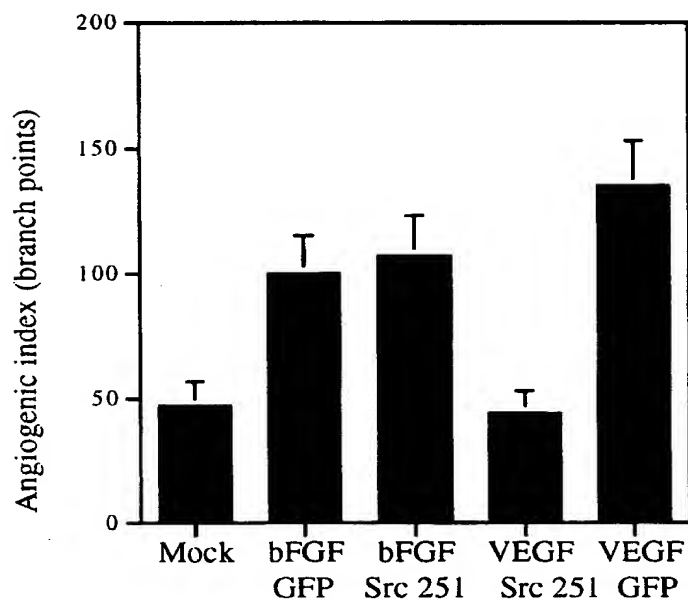
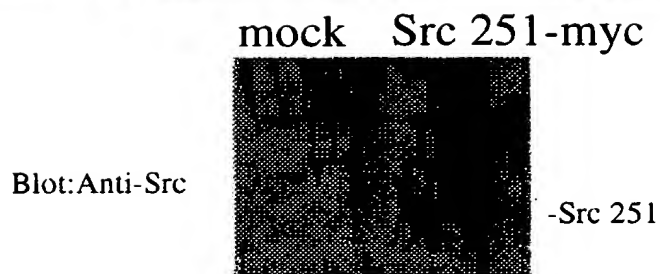


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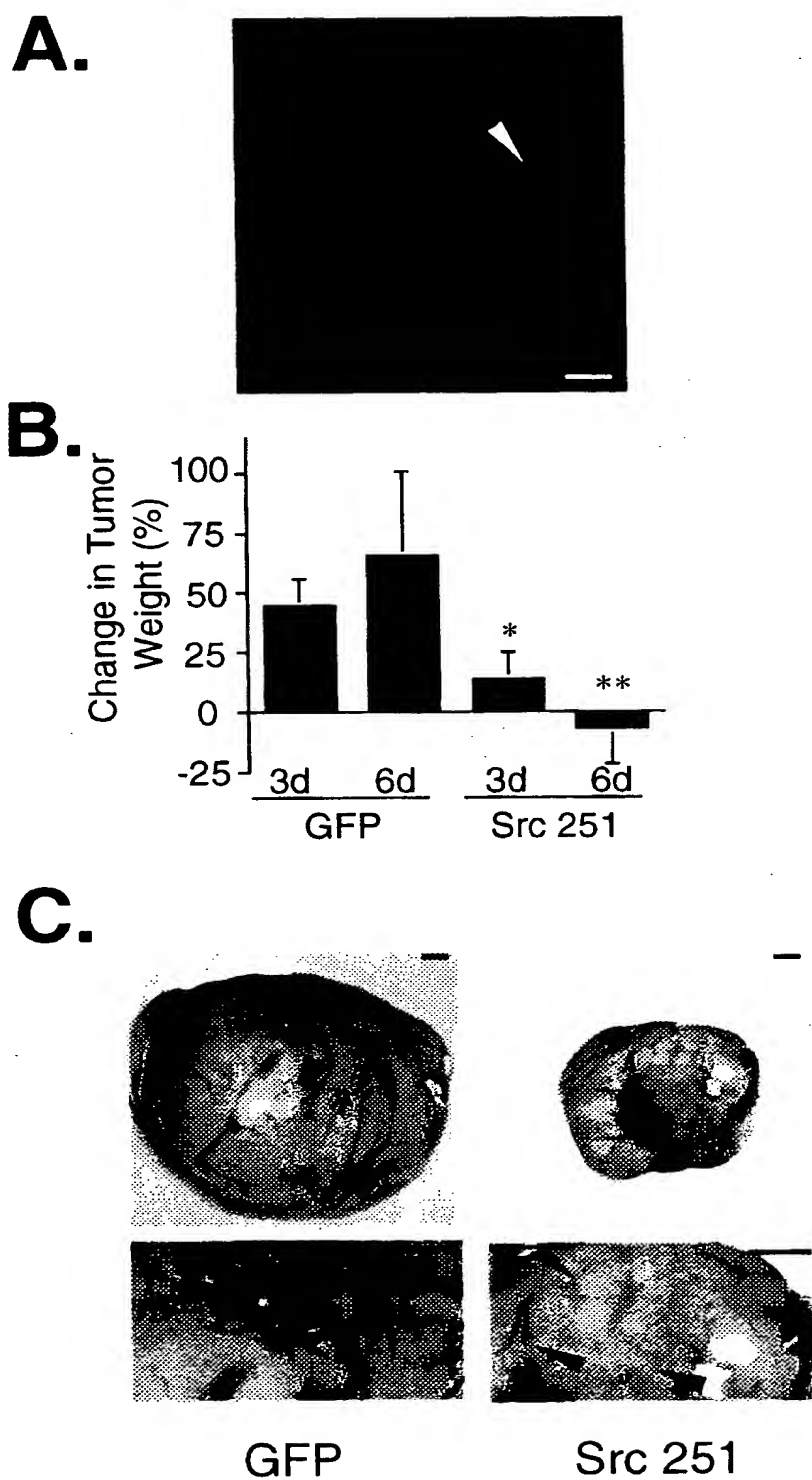


FIG. 7

# Selective requirement for Src activity during VEGF, but not bFGF-induced angiogenesis

**A.****B.****C.****FIG. 8**

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**FIG. 9**

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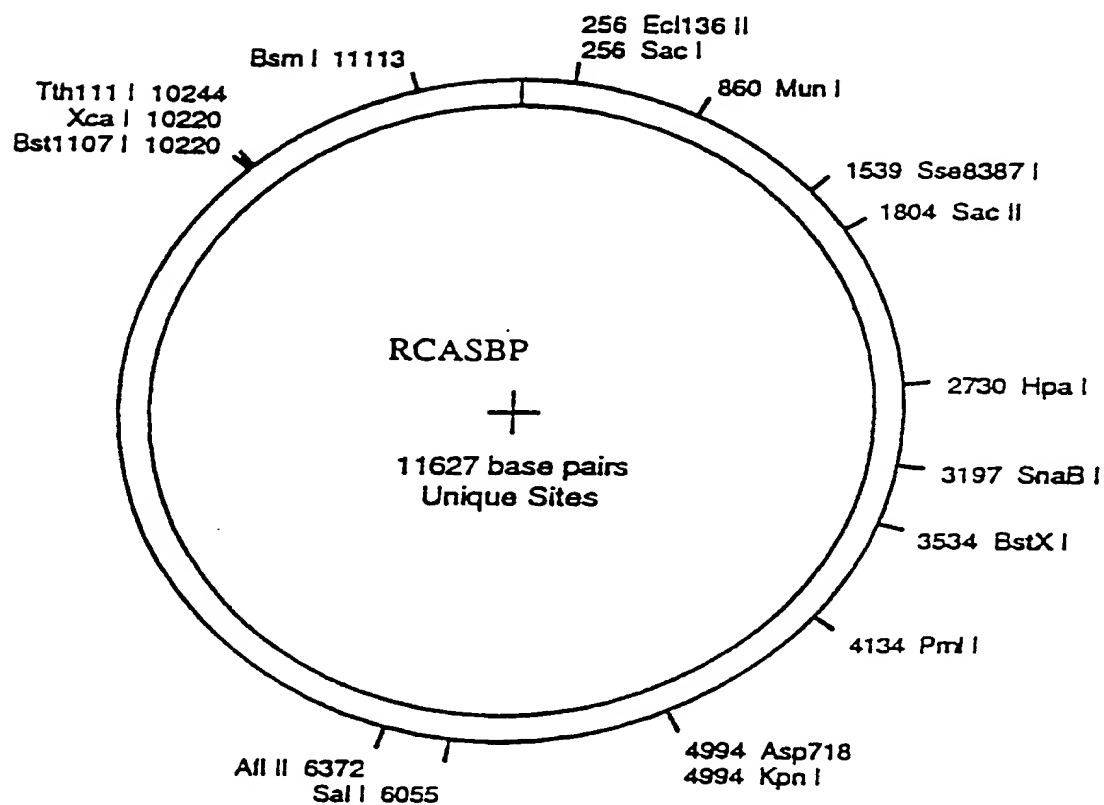


FIG. 10

## SEQUENCE LISTING

<110> The Scripps Research Institute et al.

<120> METHODS AND COMPOSITIONS USEFUL FOR MODULATION OF  
ANGIOGENESIS USING TYROSINE KINASE SRC

<130> TSRI 651.1

<140> Not yet known

<141> To be determined

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&lt;210&gt; 2

&lt;211&gt; 1759

&lt;212&gt; DNA

&lt;213&gt; Chicken

&lt;220&gt;

&lt;221&gt; gene

&lt;222&gt; (1) .. (1759)

&lt;223&gt; chicken c-SRC cDNA

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (112) .. (1710)

&lt;400&gt; 2

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Met Gly  
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gag Glu 20	cca Pro	ccc Pro	gac Asp	agc Ser	acc Thr	cac His 25	cac His	ggg Gly	gga Gly	ttc Phe	cca Pro 30	gcc Ala	tcg Ser	cag Gln	acc Thr	213
ccc Pro 35	aac Asn	aag Lys	aca Thr	gca Ala	gcc Ala 40	ccc Pro	gac Asp	acg Thr	cac His	cgc Arg 45	acc Thr	ccc Pro	agc Ser	cgc Arg	tcc Ser 50	261
ttt Phe	ggg Gly	acc Thr	gtg Val	gcc Ala 55	acc Thr	gag Glu	ccc Pro	aag Lys	ctc Leu 60	ttc Phe	ggg Gly	ggc Gly	ttc Phe	aac Asn 65	act Thr	309
tct Ser	gac Asp	acc Thr	gtt Val 70	acg Thr	tcg Ser	ccg Pro	cag Gln	cgt Arg 75	gcc Ala	ggg Gly	gca Ala	ctg Leu	gct Ala 80	ggc Gly	ggc Gly	357
gtc Val	acc Thr	act Thr	ttc Phe 85	gtg Val	gct Ala	ctc Leu	tac Tyr 90	gac Asp	tac Tyr	gag Glu	tcc Ser	cgg Arg 95	act Thr	gaa Glu	acg Thr	405
gac Asp 100	ttg Leu	tcc Ser	ttc Phe	aag Lys	aaa Lys	gga Gly 105	gaa Glu	cgc Arg	ctg Leu	cag Gln	att Ile 110	gtc Val	aac Asn	aac Asn	acg Thr	453
gaa Glu 115	ggt Gly	gac Asp	tgg Trp	tgg Trp	ctg Leu 120	gct Ala	cat His	tcc Ser	ctc Leu	act Thr 125	aca Thr	gga Gly	cag Gln	acg Thr	ggc Gly 130	501
tac Tyr	atc Ile	ccc Pro	agt Ser	aac Asn 135	tat Tyr	gtc Val	gcg Ala	ccc Pro	tca Ser 140	gac Asp	tcc Ser	atc Ile	cag Gln	gct Ala 145	gaa Glu	549
gag Glu	tgg Trp	tac Tyr	ttt Phe 150	ggg Gly	aag Lys	atc Ile	act Thr	cgt Arg 155	cgg Arg	gag Glu	tcc Ser	gag Glu	cgg Arg 160	ctg Leu	ctg Leu	597
ctc Leu	aac Asn	ccc Pro 165	gaa Glu	aac Asn	ccc Pro	cgg Arg	gga Gly 170	acc Thr	ttc Phe	ttg Leu	gtc Val	cgg Arg 175	gag Glu	agc Ser	gag Glu	645
acg Thr 180	aca Thr	aaa Lys	ggt Gly	gcc Ala	tat Tyr	tgc Cys 185	ctc Leu	tcc Ser	gtt Val	tct Ser	gac Asp 190	ttt Phe	gac Asp	aac Asn	gcc Ala	693
aag Lys 195	ggg Gly	ctc Leu	aat Asn	gtg Val	aag Lys 200	cac His	tac Tyr	aag Lys	atc Ile	cgc Arg 205	aag Lys	ctg Leu	gac Asp	agc Ser	ggc Gly 210	741
ggc Gly	ttc Phe	tac Tyr	atc Ile	acc Thr 215	tca Ser	cgc Arg	aca Thr	cag Gln	ttc Phe 220	agc Ser	agc Ser	ctg Leu	cag Gln	cag Gln 225	ctg Leu	789
gtg Val	gcc Ala	tac Tyr	tac Tyr	tcc Ser	aaa Lys	cat His	gct Ala	gat Asp 235	ggc Gly	ttg Leu	tgc Cys	cac His	cgc Arg	ctg Leu	acc Thr	837
aac	gtc	tgc	ccc	acg	tcc	aag	ccc	cag	acc	cag	gga	ctc	gcc	aag	gac	885



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Asn	Val	Cys	Pro	Thr	Ser	Lys	Pro	Gln	Thr	Gln	Gly	Leu	Ala	Lys	Asp	
		245					250					255				
gcg	tgg	gaa	atc	ccc	cgg	gag	tcg	ctg	cgg	ctg	gag	gtg	aag	ctg	ggg	933
Ala	Trp	Glu	Ile	Pro	Arg	Glu	Ser	Leu	Arg	Leu	Glu	Val	Lys	Leu	Gly	
	260					265					270					
cag	ggc	tgc	ttt	gga	gag	gtc	tgg	atg	ggg	acc	tgg	aac	ggc	acc	acc	981
Gln	Gly	Cys	Phe	Gly	Glu	Val	Trp	Met	Gly	Thr	Trp	Asn	Gly	Thr	Thr	
	275				280					285					290	
aga	gtg	gcc	ata	aag	act	ctg	aag	ccc	ggc	acc	atg	tcc	ccg	gag	gcc	1029
Arg	Val	Ala	Ile	Lys	Thr	Leu	Lys	Pro	Gly	Thr	Met	Ser	Pro	Glu	Ala	
				295					300					305		
ttc	ctg	cag	gaa	gcc	caa	gtg	atg	aag	aag	ctc	cgg	cat	gag	aag	ctg	1077
Phe	Leu	Gln	Glu	Ala	Gln	Val	Met	Lys	Lys	Leu	Arg	His	Glu	Lys	Leu	
			310					315					320			
gtt	cag	ctg	tac	gca	gtg	gtg	tcg	gaa	gag	ccc	atc	tac	atc	gtc	act	1125
Val	Gln	Leu	Tyr	Ala	Val	Val	Ser	Glu	Glu	Pro	Ile	Tyr	Ile	Val	Thr	
		325					330					335				
gag	tac	atg	agc	aag	ggg	agc	ctc	ctg	gat	ttc	ctg	aag	gga	gag	atg	1173
Glu	Tyr	Met	Ser	Lys	Gly	Ser	Leu	Leu	Asp	Phe	Leu	Lys	Gly	Glu	Met	
	340					345					350					
ggc	aag	tac	ctg	cgg	ctg	cca	cag	ctc	gtc	gat	atg	gct	gct	cag	att	1221
Gly	Lys	Tyr	Leu	Arg	Leu	Pro	Gln	Leu	Val	Asp	Met	Ala	Ala	Gln	Ile	
	355				360					365					370	
gca	tcc	ggc	atg	gcc	tat	gtg	gag	agg	atg	aac	tac	gtg	cac	cga	gac	1269
Ala	Ser	Gly	Met	Ala	Tyr	Val	Glu	Arg	Met	Asn	Tyr	Val	His	Arg	Asp	
				375					380					385		
ctg	cgg	gcg	gcc	aac	atc	ctg	gtg	ggg	gag	aac	ctg	gtg	tgc	aag	gtg	1317
Leu	Arg	Ala	Ala	Asn	Ile	Leu	Val	Gly	Glu	Asn	Leu	Val	Cys	Lys	Val	
				390				395					400			
gct	gac	ttt	ggg	ctg	gca	cgc	ctc	atc	gag	gac	aac	gag	tac	aca	gca	1365
Ala	Asp	Phe	Gly	Leu	Ala	Arg	Leu	Ile	Glu	Asp	Asn	Glu	Tyr	Thr	Ala	
		405					410					415				
cgg	caa	ggt	gcc	aag	ttc	ccc	atc	aag	tgg	aca	gcc	ccc	gag	gca	gcc	1413
Arg	Gln	Gly	Ala	Lys	Phe	Pro	Ile	Lys	Trp	Thr	Ala	Pro	Glu	Ala	Ala	
		420				425					430					
ctc	tat	ggc	cgg	ttc	acc	atc	aag	tcg	gat	gtc	tgg	tcc	ttc	ggc	atc	1461
Leu	Tyr	Gly	Arg	Phe	Thr	Ile	Lys	Ser	Asp	Val	Trp	Ser	Phe	Gly	Ile	
	435				440					445					450	
ctg	ctg	act	gag	ctg	acc	acc	aag	ggc	cgg	gtg	cca	tac	cca	ggg	atg	1509
Leu	Leu	Thr	Glu	Leu	Thr	Thr	Lys	Gly	Arg	Val	Pro	Tyr	Pro	Gly	Met	
				455					460					465		
gtc	aac	agg	gag	gtg	ctg	gac	cag	gtg	gag	agg	ggc	tac	cgc	atg	ccc	1557
Val	Asn	Arg	Glu	Val	Leu	Asp	Gln	Val	Glu	Arg	Gly	Tyr	Arg	Met	Pro	
			470					475					480			
tgc	ccg	ccc	gag	tgc	ccc	gag	tcg	ctg	cat	gac	ctc	atg	tgc	cag	tgc	1605
Cys	Pro	Pro	Glu	Cys	Pro	Glu	Ser	Leu	His	Asp	Leu	Met	Cys	Gln	Cys	
		485					490					495				
tgg	cgg	agg	gac	cct	gag	gag	cgg	ccc	act	ttt	gag	tac	ctg	cag	gcc	1653

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Trp Arg Arg Asp Pro Glu Glu Arg Pro Thr Phe Glu Tyr Leu Gln Ala  
 500 505 510  
 ttc ctg gag gac tac ttc acc tcg aca gag ccc cag tac cag cct gga 1701  
 Phe Leu Glu Asp Tyr Phe Thr Ser Thr Glu Pro Gln Tyr Gln Pro Gly  
 515 520 525 530  
 gag aac cta taggcctgga gctcctcctg gaccagagge ctcgctgtgg ggtacaggg 1759  
 Glu Asn Leu

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 <213> Chicken

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 35 40 45  
 Arg Ser Phe Gly Thr Val Ala Thr Glu Pro Lys Leu Phe Gly Gly Phe  
 50 55 60  
 Asn Thr Ser Asp Thr Val Thr Ser Pro Gln Arg Ala Gly Ala Leu Ala  
 65 70 75 80  
 Gly Gly Val Thr Thr Phe Val Ala Leu Tyr Asp Tyr Glu Ser Arg Thr  
 85 90 95  
 Glu Thr Asp Leu Ser Phe Lys Lys Gly Glu Arg Leu Gln Ile Val Asn  
 100 105 110  
 Asn Thr Glu Gly Asp Trp Trp Leu Ala His Ser Leu Thr Thr Gly Gln  
 115 120 125  
 Thr Gly Tyr Ile Pro Ser Asn Tyr Val Ala Pro Ser Asp Ser Ile Gln  
 130 135 140  
 Ala Glu Glu Trp Tyr Phe Gly Lys Ile Thr Arg Arg Glu Ser Glu Arg  
 145 150 155 160  
 Leu Leu Leu Asn Pro Glu Asn Pro Arg Gly Thr Phe Leu Val Arg Glu  
 165 170 175  
 Ser Glu Thr Thr Lys Gly Ala Tyr Cys Leu Ser Val Ser Asp Phe Asp  
 180 185 190  
 Asn Ala Lys Gly Leu Asn Val Lys His Tyr Lys Ile Arg Lys Leu Asp  
 195 200 205  
 Ser Gly Gly Phe Tyr Ile Thr Ser Arg Thr Gln Phe Ser Ser Leu Gln  
 210 215 220  
 Gln Leu Val Ala Tyr Tyr Ser Lys His Ala Asp Gly Leu Cys His Arg  
 225 230 235 240  
 Leu Thr Asn Val Cys Pro Thr Ser Lys Pro Gln Thr Gln Gly Leu Ala  
 245 250 255

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Lys Asp Ala Trp Glu Ile Pro Arg Glu Ser Leu Arg Leu Glu Val Lys  
                   260                  265                  270  
 Leu Gly Gln Gly Cys Phe Gly Glu Val Trp Met Gly Thr Trp Asn Gly  
                   275                  280                  285  
 Thr Thr Arg Val Ala Ile Lys Thr Leu Lys Pro Gly Thr Met Ser Pro  
                   290                  295                  300  
 Glu Ala Phe Leu Gln Glu Ala Gln Val Met Lys Lys Leu Arg His Glu  
                   305                  310                  315                  320  
 Lys Leu Val Gln Leu Tyr Ala Val Val Ser Glu Glu Pro Ile Tyr Ile  
                   325                  330                  335  
 Val Thr Glu Tyr Met Ser Lys Gly Ser Leu Leu Asp Phe Leu Lys Gly  
                   340                  345                  350  
 Glu Met Gly Lys Tyr Leu Arg Leu Pro Gln Leu Val Asp Met Ala Ala  
                   355                  360                  365  
 Gln Ile Ala Ser Gly Met Ala Tyr Val Glu Arg Met Asn Tyr Val His  
                   370                  375                  380  
 Arg Asp Leu Arg Ala Ala Asn Ile Leu Val Gly Glu Asn Leu Val Cys  
                   385                  390                  395                  400  
 Lys Val Ala Asp Phe Gly Leu Ala Arg Leu Ile Glu Asp Asn Glu Tyr  
                   405                  410                  415  
 Thr Ala Arg Gln Gly Ala Lys Phe Pro Ile Lys Trp Thr Ala Pro Glu  
                   420                  425                  430  
 Ala Ala Leu Tyr Gly Arg Phe Thr Ile Lys Ser Asp Val Trp Ser Phe  
                   435                  440                  445  
 Gly Ile Leu Leu Thr Glu Leu Thr Thr Lys Gly Arg Val Pro Tyr Pro  
                   450                  455                  460  
 Gly Met Val Asn Arg Glu Val Leu Asp Gln Val Glu Arg Gly Tyr Arg  
                   465                  470                  475                  480  
 Met Pro Cys Pro Pro Glu Cys Pro Glu Ser Leu His Asp Leu Met Cys  
                   485                  490                  495  
 Gln Cys Trp Arg Arg Asp Pro Glu Glu Arg Pro Thr Phe Glu Tyr Leu  
                   500                  505                  510  
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&lt;223&gt; human c-SRC cDNA

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (134)..(1483)

&lt;400&gt; 4

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agctcctgag aag atg tca gca ata cag gcc gcc tgg cca tcc ggt aca 169
      Met Ser Ala Ile Gln Ala Ala Trp Pro Ser Gly Thr
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gaa tgt att gcc aag tac aac ttc cac ggc act gcc gag cag gac ctg 217
Glu Cys Ile Ala Lys Tyr Asn Phe His Gly Thr Ala Glu Gln Asp Leu
      15                    20                      25

ccc ttc tgc aaa gga gac gtg ctc acc att gtg gcc gtc acc aag gac 265
Pro Phe Cys Lys Gly Asp Val Leu Thr Ile Val Ala Val Thr Lys Asp
      30                    35                      40

ccc aac tgg tac aaa gcc aaa aac aag gtg ggc cgt gag ggc atc atc 313
Pro Asn Trp Tyr Lys Ala Lys Asn Lys Val Gly Arg Glu Gly Ile Ile
      45                    50                      55                      60

cca gcc aac tac gtc cag aag cgg gag ggc gtg aag gcg ggt acc aaa 361
Pro Ala Asn Tyr Val Gln Lys Arg Glu Gly Val Lys Ala Gly Thr Lys
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ctc agc ctc atg cct tgg ttc cac ggc aag atc aca cgg gag cag gct 409
Leu Ser Leu Met Pro Trp Phe His Gly Lys Ile Thr Arg Glu Gln Ala
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Glu Arg Leu Leu Tyr Pro Pro Glu Thr Gly Leu Phe Leu Val Arg Glu
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agc acc aac tac ccc gga gac tac acg ctg tgc gtg agc tgc gac ggc 505
Ser Thr Asn Tyr Pro Gly Asp Tyr Thr Leu Cys Val Ser Cys Asp Gly
      110                    115                      120

aag gtg gag cac tac cgc atc atg tac cat gcc agc aag ctc agc atc 553
Lys Val Glu His Tyr Arg Ile Met Tyr His Ala Ser Lys Leu Ser Ile
      125                    130                      135                      140

gac gag gag gtg tac ttt gag aac ctc atg cag ctg gtg gag cac tac 601
Asp Glu Glu Val Tyr Phe Glu Asn Leu Met Gln Leu Val Glu His Tyr
      145                    150                      155

acc tca gac gca gat gga ctc tgt acg cgc ctc att aaa cca aag gtc 649
Thr Ser Asp Ala Asp Gly Leu Cys Thr Arg Leu Ile Lys Pro Lys Val
      160                    165                      170

atg gag ggc aca gtg gcg gcc cag gat gag ttc tac cgc agc ggc tgg 697
Met Glu Gly Thr Val Ala Ala Gln Asp Glu Phe Tyr Arg Ser Gly Trp
      175                    180                      185

gcc ctg aac atg aag gag ctg aag ctg ctg cag acc atc ggg aag ggg 745
Ala Leu Asn Met Lys Glu Leu Lys Leu Leu Gln Thr Ile Gly Lys Gly
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gag ttc gga gac gtg atg ctg ggc gat tac cga ggg aac aaa gtc gcc 793

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Glu 205	Phe	Gly	Asp	Val	Met 210	Leu	Gly	Asp	Tyr	Arg 215	Gly	Asn	Lys	Val	Ala 220		
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gcc Ala	tca Ser	gtc Val	atg Met 240	acg Thr	caa Gln	ctg Leu	cgg Arg	cat His 245	agc Ser	aac Asn	ctg Leu	gtg Val	cag Gln 250	ctc Leu	ctg Leu	889	
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16/18

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18/18

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/11780

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 9/00

US CL : 435/183; 514/2

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/183; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MUKHOPADHYAY et al. Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation. Nature. June 1995, Vol. 375, pages 577-581, especially abstract, page 578, col. 2, para. 2 through page 580, and Figs 3 and 4.	1-30 and 38
Y	KAPLAN et al. Association of the amino-terminal half of c-Src with focal adhesions alters their properties and is regulated by phosphorylation of tyrosine 527. EMBO J. 1994, Vol 13, pages 4745-4756, entire document	1-30, 37, 38

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

Date of mailing of the international search report

18 OCT 1999

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/11780

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MUKHOPADHYAY et al. Wild-type p53 and v-Src exert opposing influences on human vascular endothelial growth factor gene expression. Cancer Research. 15 December 1995, Vol. 55, pages 6161-6165.	1-30, 38
Y	FLORIO et al. Aberrant protein phosphorylation at tyrosine is responsible for the growth inhibitory action of pp60v <sup>src</sup> expressed in the yeast saccharomyces cerevisiae. American Society For Cell Biology. March 1994, Vol. 5, pages 283-296, especially abstract	5-16, 21-30, 38
Y	US 5,264,618 A (FELGNER et al) 23 November 1993, col. 18, lines 18-33; and col. 20, lines 17-25.	14
Y	KOEGL et al. Generation of a temperature-sensitive cSRC. Virology. 1993, Vol. 196, pages 368-371, especially abstract.	1, 3, 12-16, and 37
Y	HIRAI et al. SH2 mutants of c-src that are host dependent for transformation are trans-dominant inhibitors of mouse cell transformation by activated c-src. Genes and Development. December 1990, Vol. 4, No. 12B, pages 2342-2352, abstract only.	1,3,12-16, 37

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/11780

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-30, 37, 38

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/11780

### B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

#### MEDLINE

Src, K295M, Y527F, angiogenesis, (angiogen?(10w)(stimul? or generat? or caus? or improv? or therap?))

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-30, 37 and 38, drawn to a src protein and a method of use.

Group II, claim(s) 1-16, drawn to a nucleic acid encoding a src protein.

Group III, claims 17-32, drawn to and methods of using nucleic acids encoding a src protein.

Group IV, claim 33, drawn to a viral gene transfer vector encoding a src protein.

Group V, claim 34, drawn to a non-viral gene transfer vector encoding a src protein.

Group VI, claim 35, drawn to a viral gene transfer vector encoding a src protein lacking kinase activity.

Group VII, claim 36, drawn to a non-viral gene transfer vector encoding a src protein lacking kinase activity.

The inventions listed as Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Claims 1-16 are drawn to a protein and a nucleic acid. PCT rule 13, 37 C.F.R. 1.475(c,d) does not allow for claims drawn to multiple independent products. If multiple products are claimed, the first invention of the category first mentioned in the claims, and the first recited invention of each of the other categories related to the first invention will be considered as the main invention. See PCT Article 17(3)(a) and 37 C.F.R. 1.476(c).

The technical feature of group I is the Src protein of claim 1. Group I is therefore composed of claims to the Src protein (claims 1-16, 37 and 38), and methods of using it (claims 17-30). The Src protein of claim 1, and genes encoding it, are well known in the art (see for example, Bricknell, Crit Rev Oncog 3: 401-446, 1992, abstract), therefore the claims have no special technical feature.

Groups II-VII are linked to group I because they comprise polynucleotides encoding the protein which is the technical feature of group I. However, because there can be no special technical feature, there is a lack of unity between the groups.

## PATENT COOPERATION TREATY

PCT

## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents  
 United States Patent and Trademark  
 Office  
 Box PCT  
 Washington, D.C. 20231  
 ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 06 March 2000 (06.03.00)	
International application No. PCT/US99/11780	Applicant's or agent's file reference tsri6511
International filing date (day/month/year) 28 May 1999 (28.05.99)	Priority date (day/month/year) 29 May 1998 (29.05.98)
Applicant CHERESH, David, A. et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:  
 15 December 1999 (15.12.99)

☐ in a notice effecting later election filed with the International Bureau on:  
 \_\_\_\_\_

2. The election ☒ was

☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer Diana Nissen Telephone No.: (41-22) 338.83.38
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**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A61K 31/70, C07H 21/00</b>		<b>A1</b>	(11) International Publication Number: <b>WO 98/19686</b>
			(43) International Publication Date: 14 May 1998 (14.05.98)
(21) International Application Number: PCT/US97/20404			(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
(22) International Filing Date: 7 November 1997 (07.11.97)			
(30) Priority Data: 60/030,358 8 November 1996 (08.11.96) US 08/884,866 30 June 1997 (30.06.97) US			
(71) Applicant (for all designated States except US): REGENTS OF THE UNIVERSITY OF CALIFORNIA, SAN DIEGO [US/US]; 9500 Gilman Drive, La Jolla, CA 92093-0919 (US).			
(72) Inventors; and (75) Inventors/Applicants (for US only): CHIEN, Shu [US/US]; 9445 La Jolla Farms Road, La Jolla, CA 92037 (US). SHYY, John, Y.-J. [US/US]; 1287 Via Terceto, San Diego, CA 92130 (US).			
(74) Agent: FAWCETT, Robroy, R.; Gray Cary Ware & Freidenrich, Suite 1600, 4365 Executive Drive, San Diego, CA 92121 (US).			Published With international search report.
(54) Title: THERAPEUTIC METHODS FOR VASCULAR INJURY			
(57) Abstract			
<p>The present invention comprises methods of treating disorders associated with vascular injury from mechanical stimuli including restenosis, atherosclerosis and reperfusion injury. In one embodiment of the invention gene therapy techniques are applied using genes encoding a variety of proteins that play key roles in transducing an extracellular signal through to the nucleus including src, Ras, MEKK and JNK. These proteins are mutated such that they are rendered signal transduction incompetent, thus abrogating their ability to induce a cellular response. The invention further encompasses viral gene therapy vectors containing genes encoding these signaling incompetent mutants and pharmaceutical compositions. Additional embodiments of the invention encompass alternative means of inhibiting the key signal transduction pathways related to mechanical injury. One alternative includes the use of antisense versions of genes encoding key proteins such as src, Ras, MEKK, JNK and the like. Chemical compounds acting as enzymatic inhibitors or disrupters of protein:protein interactions are also contemplated by the present invention.</p>			
<p>102 against Linking element 17</p>			

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## THERAPEUTIC METHODS FOR VASCULAR INJURY

### Field of the Invention

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The present invention relates to methods to prevent or mitigate vascular injury caused by various sources of mechanical stimuli, such as fluid shear stress or balloon angioplasty, and associated chemical factors.

10 Also disclosed are pharmaceutical compositions comprising signal transduction incompetent mutants for use in gene therapy to treat vascular injury related disorders such as restenosis.

15

### Background of the Invention

Blood vessels are composed of a variety of cell types including endothelial cells (ECs) and smooth muscle cells (SMCs). Vascular endothelial cells (ECs), located  
20 at the interface between the blood and the vessel, are exposed to the mechanical environment resulting from hemodynamic activities. SMCs are generally only exposed to fluid shear stress after injury to the vessel wall, such as after angioplasty or introduction of an  
25 intravascular stent. The hemodynamic force resulting from fluid shear stress plays a significant role in a



variety of serious disorders including restenosis, atherosclerosis and reperfusion injury.

The application of shear stress on the vessel wall induces a number of morphological and functional changes in the vascular endothelium. Shear stress has been reported to induce the expression of a variety of genes including those encoding growth factors, (e.g., platelet derived growth factor and transforming growth factor  $\beta$ -1), vasoconstrictors (e.g., endothelin-1), vasodilators (e.g., nitric oxide synthase), adhesion molecules (e.g., intercellular adhesion molecule-1, ICAM-1), and monocyte chemoattractants (e.g., monocyte chemotactic protein-1, MCP-1) (for review see, Davies, P. F., *Physiol Rev.*, 75:519560, 1995). It is, as yet, not known precisely where and how mechanical stimuli are transduced into biochemical signals.

Cellular signal transduction is a fundamental mechanism whereby external stimuli that regulate diverse cellular processes are relayed to the interior of cells. Signal transduction events often result in nuclear transcription and the production of new proteins. One of the key biochemical mechanisms of signal transduction involves the reversible phosphorylation of proteins, which enables regulation of the activity of mature proteins by altering their structure and function. The best characterized protein kinases in eukaryotes

phosphorylate proteins on the alcohol moiety of serine, threonine and/or tyrosine residues. Phosphorylated amino acids often act as protein:protein interaction sites allowing two proteins to become associated in some  
5 functionally significant manner.

Signal transduction molecules include both those that have some catalytic function and those that serve primarily as adaptor proteins. Adaptor proteins have no catalytic activity and are composed largely of  
10 stretches of amino acids that function as protein:protein interaction sites. Commonly known protein:protein interaction domains include the SH2 (Src Homology 2) domain (Sadowski, et al, Mol. Cell. Biol. 6:4396, 1986; Pawson and Schlessinger, Curr. Biol. 3:434, 1993), the  
15 SH3 domain (Mayer, et al, Nature 332:272, 1988; Pawson and Schlessinger, Curr. Biol. 3:434, 1993), and pleckstrin (PH) domain (Ponting, TIBS 21:245, 1996; Haslam, et al, Nature 363:309, 1993)

A major process through which extracellular  
20 stimuli can be transmitted into cells involves membrane-associated p21<sup>ras</sup> and its downstream cytoplasmic kinase pathways, especially the members in the mitogen-activated protein kinase (MAPK) family. p21<sup>ras</sup> is a small GTPase molecule that plays a key role in the signal transduction  
25 pathways of cellular responses to stimuli by mitogens, cytokines, environmental stresses, and UV irradiation.

p21<sup>ras</sup> cycles between an active GTP-bound state and an inactive GDP-bound state, thereby functioning as a molecular switch in response to extracellular stimuli in the control of normal and transformed cell growth.

- 5 Activated p21<sup>ras</sup> triggers two protein kinases, Raf-1 and MEK (MAPK kinase) kinase (MEKK) which activate the downstream MAPKS, including c-Jun NH<sub>2</sub>-terminal kinases (JNK) and a cellular signal-regulated kinases ERK (see Figure 1). DeVries-Smits, *et al*, *Nature*, 357:602-604, 10 1992; Marshall, C.J., *Cell*, 80:179-185, 1995. Raf-1 activates ERK but not JNK, whereas MEKK preferentially mediates the activity of JNK over ERK. Marshall, C.J., *Cell*, 80:179-185, 1995; Wang, *et al*, *Science*, 260:1124-1127, 1993. Ras-related signal transduction has been 15 linked to a wide variety of cellular functions including proliferation and release of inflammatory cytokines. Inappropriate Ras activity has been associated with tumor formation.

- A number of investigators have suggested a 20 connection between fluid shear stress and Ras-related signal transduction. However it remains unclear which of the many signal transduction pathways involving Ras is related to the undesirable effects of fluid shear stress. A need still exists for a clear understanding of the 25 pathways involved and a means for translating this knowledge in therapeutic approaches for the treatment of

fluid shear stress related disorders in patients. The present invention addresses these needs.

### Brief Description of the Invention

5

The present invention comprises methods of treating disorders associated with vascular injury from mechanical stimuli including restenosis, atherosclerosis and reperfusion injury. In one embodiment of the invention gene therapy techniques are applied using genes encoding a variety of proteins that play key roles in transducing an extracellular signal through to the nucleus including src, Ras, MEKK and JNK. These proteins are mutated such that they are rendered signal transduction incompetent, thus abrogating their ability to induce a cellular response. The invention further encompasses viral gene therapy vectors containing genes encoding these signaling incompetent mutants and pharmaceutical compositions.

15

Additional embodiments of the invention encompass alternative means of inhibiting the key signal transduction pathways related to mechanical injury. One alternative includes the use of antisense versions of genes encoding key proteins such as src, Ras, MEKK, Jnk and the like. Chemical compounds acting as enzymatic

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inhibitors or disrupters of protein:protein interactions are also contemplated by the present invention.

Mechanical injury induces a variety of transient responses in the cellular cytoplasm and nucleus, however, there is little knowledge of how cells transduce mechanical stimuli into biochemical signals which ultimately activate downstream gene expression. The present inventors have found that mechanical injury in the form of fluid shearing activates p21<sup>ras</sup> in ECs in a rapid and transient manner, and that this activation is followed by the activation of the MEKK - JNK pathway. In contrast, the ERK pathway is weakly activated by fluid shearing and is not essential for the shear-induced activation transcription.

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#### Brief Description of the Figures

FIG 1 schematically illustrates the signal transduction pathways involving fluid shear induction of Ras that are relevant to the invention.

FIG 2 shows that fluid shearing increases the ratio of p21<sup>ras</sup>-GTP/p21<sup>ras</sup>GDP. The results represent the mean  $\pm$  SD from three studies.

FIG. 3A illustrates that RasN17 abolishes the shear-induced AP-1/TRE activation. Dark bars indicate sheared samples and open bars are static controls. The

results represent the mean  $\pm$  SD from at least three studies.

FIG. 3B shows the results of the assay illustrated in FIG. 3A wherein the Ras mutants are  
5 cotransfected with MCP1-Luc-540 instead of 4xTRE-P1-Luc. Dark bars indicate sheared samples and open bars are static controls.

FIG. 4A shows that mutants JNK(K-R) and MEKK(K-M) attenuate the shear-induced AP-1/TRE  
10 activation. The results represent the mean  $\pm$  SD from six experiments.

FIG. 4B shows the results of the assay illustrated in FIG. 4A wherein the mutants are cotransfected with MCP1-Luc-540 instead of 4xTRE-P1-Luc.

15 FIG. 5 demonstrates that Raf-1 and ERK are not required for the shear-induced AP-1/TRE. The results represent the mean  $\pm$  SD from six experiments.

FIG. 6A illustrates that fluid shearing increases the transcriptional activity of c-Jun and  
20 appropriate mutants attenuate this shear-induced activity. The results represent the mean  $\pm$  SD from six experiments.

FIG. 6B illustrates that fluid shearing increases the transcriptional activity of c-Jun and  
25 appropriate mutants attenuate this shear-induced

activity. The results represent the mean $\pm$ SD from six experiments.

FIG. 7 shows that the induction of luciferase activity by serum or PDGF is significantly attenuated by cotransfection with RasN17.

FIG. 8A shows that Ad-RasN17 attenuates the mitotic responses of PSMCs to serum. The hatched bars are 15% serum and the open bars are 0.5% serum.

FIG. 8B illustrates that Ad-RasN17 attenuates the mitotic responses of PSMCs to PDGF. The hatched bars are 10 ng/ml PDGF and the open bars are 0 ng/ml PDGF.

FIG. 8 presents results of luciferase assay showing that RasN17 inhibits the serum- and PDGF-induced AP-1/TRE in bovine aortic endothelial cells. Open bars represent controls and dark bars are assays with RasN17.

FIG. 9 shows that the administration of RasN17 into rat common carotid arteries markedly reduced the restenosis of the vessel after balloon injury in vivo.

FIG. 10A compares kinase assays of HA-JNK1 co-transfected with either pGL2 as an empty plasmid (lanes 1 and 2) or v-src(K295R) (lanes 3 and 4) into BAEC in a T-75 tissue culture flask. Bar graph shows the relative kinase activity (mean $\pm$ SD) from 3 sets of experiments. Open bars are static controls

and dark bars are sheared samples. Asterix indicates that the difference is significant ( $p < 0.05$ ) between lanes 2 and 4.

FIG. 10B compares kinase assays of HA-JNK1 co-transfected with c-src(wt) (lanes 1 and 2) or c-src(K295R) (lanes 3 and 4). Open bars are static controls and dark bars are sheared samples. Asterix indicates that the difference is significant ( $p < 0.05$ ) between lanes 2 and 4.

FIG. 10C compares kinase assays of HA-JNK1 co-transfected with pGL2 as an empty plasmid (lane 1), 6 mg of c-src(F527) (lane 2), c-src(F527) and RasN17 (lane 3), RasL61 (lane 4), or RasL61 and v-src(K295R) (lane 5). Open bars are static controls and dark bars are sheared samples. Asterix indicates that the difference is significant ( $p < 0.05$ ) between lanes 2 and 4.

#### Detailed Description of the Invention

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The present invention comprises methods of inhibiting the response of vascular cells to mechanical injury. In particular the methods of the invention allow for the treatment of vascular disorders, such as restenosis, by inhibiting activation of a Ras signal

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transduction pathway induced by mechanical injury.

"Mechanical injury" as used herein refers to injury to the vascular cell wall from mechanical sources such as angioplasty, atherectomy, insertion of a stent, 5 resumption of blood flow following ischemia, and the like. Mechanical injury is often associated with fluid shear stress, thus these terms are used interchangeably herein.

Presently preferred methods of the invention 10 involve the use of one or more proteins that have been modified such that they are incapable of propagating a signal as part of a signal transduction pathway. The proteins can be modified in any of a variety of ways to render them signal transduction incompetent, such as by 15 making them catalytically inactive or incapable of forming a protein:protein interaction with another component of the signal transduction pathway. Such mutant proteins are introduced into a patient using gene therapy methods.

20 Also contemplated by the invention is the use of antisense technology. Antisense versions of genes encoding key signal transduction proteins are introduced into the cells of a patient to be treated. These cells then become incapable of synthesizing the target 25 protein(s), thus rendering them incapable of responding to fluid shear stress in an undesired manner.

In an alternative embodiment of the invention, the relevant signal transduction pathways are inhibited using organic chemical compounds. Such compounds can be inhibitors of catalytic activity (such as kinase  
5 inhibitors) or inhibitors of protein:protein interactions. Exemplary catalytic inhibitors suitable for use in the practice of the invention include quinazolines, quinoxalines, tyrphostins, indolinones, and the like. Examples of suitable inhibitors of  
10 protein:protein interactions include, for example, asteriquinones. Examples of suitable Ras inhibitors include cylindrols, actinoplanic acids, and the like.

Ras is a membrane-associated GTPase that plays a central role in a complex set of signal transduction  
15 pathways. One of the most widely studied of these pathways leads from an extracellular ligand to transcription of genes in the nucleus via Ras. The extracellular ligand, for example a growth factor like platelet-derived growth factor (PDGF), binds to its cell  
20 membrane-spanning receptor, which in the case of PDGF stimulates the phosphorylation of amino acids on the intracellular part of the receptor. The phosphorylated receptor provides an interaction site for the adaptor protein Grb2 (growth factor binding protein 2). Grb2 is  
25 constitutively bound to Sos (son of sevenless), a guanine nucleotide-exchange factor, and binding of the Grb2:Sos

complex to the phosphorylated receptor brings it into close contact Ras-GDP, which is associated with the inner surface of the cell membrane. This causes Ras to release GDP in exchange for GTP, creating the active form of Ras.

5 Activated Ras then binds to and activates a kinase called Raf, which in turn binds to and activates MEK. MEK thereupon activates Erks 1 and 2 (also known as MAP kinases), which in turn activates many proteins, including a variety of transcription factors. Thus is a

10 signal transduced from the extracellular space through the cytoplasm to the nucleus.

Ras participates in several other signal transduction pathways as well. In one, Ras activates MEKK, which in turn activates Jnk kinase. Jnk kinase

15 phosphorylates and activates Jnk, which subsequently activates a variety of transcription factors including c-jun, AP-1, TRE, Elk-1, and the like. For the purposes of this disclosure, this pathway is referred to as the "Jnk pathway" whereas the preceding pathway is referred to as

20 the "Erk pathway". Ras can also transduce a signal through the GTPases Rac and Rho, although the precise mechanism is still unresolved.

A variety of upstream stimuli can result in Ras activation including the binding of growth factors and

25 cytokines to their receptors and UV radiation. The inventors have shown that fluid shear stress can also

induce the activation of Ras. Suprizingly, however, shear stress activation of Ras results in the preferential activation of the Jnk pathway compared to the Erk pathway. Thus shear stress-related disorders can  
5 be treated by reducing Jnk pathway signaling.

The inventors have also found that shear stress activates src, a non-receptor kinase generally localized to the cell membrane. Src is known to phosphorylate a variety of substrates and can interact with Grb2:Sos.  
10 Importantly, the inventors have demonstrated that the shear stress-induced activation of Src eventually results in the activation of the Jnk pathway and that introduction of a signaling incompetent src into a shear stress stimulated cell abrogates Jnk pathway activation.  
15 Thus signaling molecules upstream of Ras are additional therapeutic targets

Proteins involved in Ras signal transduction, and therefore suitable for creating signaling incompetent mutants useful in the practice of the invention, include  
20 proteins both upstream and downstream of Ras. Specific examples include focal adhesion kinase (Fak), Grb2, Sos, Shc, Rac, Rho, I kappa B kinase (IKK), Cdc42, Jun N-terminal kinase (Jnk, also known as stress activated kinase of SAPK), JNK kinase (JNKK), MAP kinase/ERK-  
25 activating kinase (MEK), and MEK kinase (MEKK), and the like. General reviews of Ras signal transduction

pathways can be found in Wiesmeuller and Wittinghofer, Cellular Signaling 6(3):247-267, 1994 and Joneson and Bar-Sagi, J Mol Med 75:587-593, 1997, both of which are incorporated herein by reference in their entirety.

5           In one aspect, the present invention demonstrates that the use of signal transduction incompetent mutants of proteins active in Ras-mediated signal transduction, separately or in combination, operate(s) to essentially abolish shear-induced  
10 activation of the Jnk signal transduction pathway. As a result, concomitant transient responses, in both the cytoplasm and the nucleus, to shear stress are abrogated. Although these proteins are well known to those in the art, their link to fluid shear stress and the use of  
15 signaling incompetent mutants thereof to abrogate their activities has yet to be described.

          The mutants of the invention, in the form of locally delivered DNA or peptides, can be used *in vivo* to prevent or reduce the severity of restenosis following  
20 transluminal percutaneous angioplasty (TCPA), or to treat other disorders associated with fluid shear stress such as reperfusion injury.

          The signal transduction incompetent mutants of the invention are produced by methods well known to those  
25 skilled in the art. Among the mutants contemplated by the invention are those that lack one or more of the

following functions: catalytic activity and interaction with other components of the signal transduction pathway.

Signaling incompetent mutants can be produced by a variety of methods including one or more point  
5 mutations, deletion of one or more amino acids essential for one of the functions listed above or addition of one or more amino acids such that the normal structure and function of the protein so modified is disturbed.

Sequences employed throughout the present  
10 specification include reference to particular amino acids. As will be recognized by those of skill in the art, each of the amino acids may be encoded by several different nucleic acid triplet codons. A mutation resulting in a single nucleic acid change (a point  
15 mutation) can therefore result in the encoding of different amino acid. These various nucleic acid triplet codons are noted in Table 1.

Table 1

First Pos. (5') (3')	Second Pos.				Third pos.
	U	C	A	G	
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
10	Leu	Ser	Stop	Stop	A
	Leu	Ser	Stop	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
15	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met*	Thr	Lys	Arg	G
20	G	Val	Ala	Asp	U
		Val	Ala	Asp	C
		Val	Ala	Glu	A
		Val*	Ala	Glu	G

\*Codons used for transcription initiation

Amino acids can be represented by a three letter or a single letter abbreviation of their name. Both of these designations are shown in Table 2 below.

Table 2

G - Glycine (Gly)	Q - Glutamine (Gln)
P - Proline (Pro)	N - Asparagine (Asn)
A - Alanine (Ala)	E - Glutamic Acid (Glu)
V - Valine (Val)	R - Arginine (Arg)
L - Leucine (Leu)	D - Aspartic Acid (Asp)
I - Isoleucine (Ile)	S - Serine (Ser)
M - Methionine (Met)	T - Threonine (Thr)
C - Cysteine (Cys)	K - Lysine (Lys)
F - Phenylalanine (Phe)	H - Histidine (His)
Y - Tyrosine (Tyr)	W - Tryptophan (Trp)

Particular signal transduction mutants useful in practicing the invention include src (K-R), Ras N17 (Feig and Cooper, Mol Cell Biol 8:3235-3243, 1988), L61, S186 (Medema, et al, Mol Cell Biol 11:5963-5067, 1991), MEKK (K-R),  $\Delta$ mSOS1, FAK(F397Y), and JNK (K-R) as discussed in greater detail in the Examples below.

The invention further provides for mutant nucleic acid gene sequences encoding the mutant signal transduction incompetent proteins discussed above. These gene sequences are preferably contained within an expression vector suitable for use in gene therapy.

Suitable expression vectors for use in the invention include those derived from viruses such as retroviruses, vaccinia virus, adenovirus, adeno-associated virus, herpes viruses, other RNA viruses, or bovine papilloma virus, and the like. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors containing coding sequences for the mutant genes of the invention. See, for example, the techniques described in Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. (1989), and in Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y. (1989).

A vector having nucleic acid sequences encoding a mutant useful in the invention is additionally provided



in which the nucleic acid sequence is expressed only in specific tissue. Methods of achieving tissue-specific gene expression are known in the art, as set forth for example in International Publication No. WO 93/09236, 5 filed November 3, 1992, published May 13, 1993 and incorporated by reference herein.

It has been shown that adenovirus proteins are capable of destabilizing endosomes and enhancing the uptake of DNA into cells. The admixture of adenovirus to 10 solutions containing DNA complexes, or the binding of DNA to polylysine covalently attached to adenovirus using protein crosslinking agents substantially improves the uptake and expression of the recombinant gene. (Curiel, et al., Am. J. Respir. Cell. Mol. Biol., 6:247-52, 15 1992). Thus the use of adenovirus gene therapy vectors is particularly advantageous in the practice of the invention.

Alternatively, recombinant nucleic acid molecules encoding protein sequences can be used as naked 20 DNA or in reconstituted systems e.g., liposomes or other lipid systems for delivery to target cells (See e.g., Felgner et al., Nature 337:387-8, 1989). Several other methods for the direct transfer of plasmid DNA into cells exist for use in human gene therapy and involve targeting 25 the DNA to receptors on cells by complexing the plasmid DNA to proteins (see, Miller, Nature 357:455-460, 1992).

As used herein "gene therapy" is a form of gene transfer and is included within the definition of gene transfer as used herein and specifically refers to gene transfer to express a therapeutic product from a cell *in vivo* or *in vitro*. The basic science of gene therapy is described in Mulligan, Science 260:926-931, (1993). Gene transfer can be performed *ex vivo* on cells which are then transplanted into a patient, or can be performed by direct administration of the nucleic acid or nucleic acid-protein complex into the patient.

As used herein "gene transfer" means the process of introducing a foreign nucleic acid molecule into a cell. Gene transfer is commonly performed to enable the expression of a particular product encoded by the gene. The product may include a protein, polypeptide, antisense DNA or RNA, or enzymatically active RNA. Gene transfer can be performed in cultured cells or by direct administration into animals. Generally gene transfer involves the process of nucleic acid contact with a target cell by non-specific or receptor mediated interactions, uptake of nucleic acid into the cell through the membrane or by endocytosis, and release of nucleic acid into the cytoplasm from the plasma membrane or endosome. Expression may require, in addition, movement of the nucleic acid into the nucleus

of the cell and binding to appropriate nuclear factors for transcription.

In its simplest form, gene transfer can be performed by simply injecting minute amounts of DNA into the nucleus of a cell, through a process of microinjection. (Capecchi MR, Cell 22:479-88, 1980). Once recombinant genes are introduced into a cell, they can be recognized by the cell's normal mechanisms for transcription and translation, and a gene product will be expressed. Other methods have also been attempted for introducing DNA into larger numbers of cells. These methods include: transfection, wherein DNA is precipitated with calcium phosphate and taken into cells by pinocytosis (Chen C. and Okayama H, Mol. Cell Biol. 7:2745-52, 1987); electroporation, wherein cells are exposed to large voltage pulses to introduce holes into the membrane (Chu G., et al., Nucleic Acids Res., 15:1311-26, 1987); lipofection/liposome fusion, wherein DNA is packaged into lipophilic vesicles which fuse with a target cell (Felgner PL., et al., Proc. Natl. Acad. Sci. USA. 84:7413-7, 1987); and particle bombardment using DNA bound to small projectiles (Yang NS. et al., Proc. Natl. Acad. Sci. 87:9568-72, 1990), and the like. Another method for introducing DNA into cells is to couple the DNA to chemically modified proteins.

Delivery methods particularly useful in the practice of the invention include the deposition of the gene therapy vector on a stent placed in a vessel with the object of decreasing restenosis and reclosing of the vessel. This technique is described in Ye, et al, ASAIO J 42:M823-M827, 1996 and US 5,562,922, issued to Lambert October 8, 1996, both incorporated by reference herein.

Alternatively, gene therapy vectors comprising the mutants of the present invention can be delivered during or immediately after balloon angioplasty using techniques well known in the art. Such techniques are described in, for example, Gottsauner-Wolf, et al, Cathet Cardiovasc Diagn 42:102,108, 1997; Isner, et al, Hum Gene Ther 7:959-988, 1996, both incorporated by reference herein, and in the Examples below.

In the case of chemical compounds, the particular compounds selected for use in treatment can be administered to a patient either by itself or in a pharmaceutical composition where it is mixed with suitable carriers or excipient(s). In treating a patient, a therapeutically effective dose of the substance is administered. A therapeutically effective dose refers to that amount of the substance that results in amelioration of symptoms or a prolongation of survival in a patient.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell culture or experimental animals. Cell culture assays and animal studies can be used for

5 determining the  $LD_{50}$  (the dose lethal to 50% of a population) and the  $ED_{50}$  (the dose therapeutically effective in 50% of a population). The dose ratio between toxic and therapeutic effects is the therapeutic index, which can be expressed as the ratio  $LD_{50}/ED_{50}$ .

10 Compounds which exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosages for use in humans. The dosage of such compounds lies preferably within a range of circulating

15 concentrations that include the  $ED_{50}$  with little or no toxicity. The dosage may vary within this range depending upon a variety of factors, e.g., the dosage form employed, the route of administration utilized, the condition of the subject, and the like. The exact

20 formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g. Fingl et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 pl).

Depending on the specific conditions being

25 treated, such agents may be formulated and administered systemically or locally. Techniques for formulation and

administration may be found in "Remington's Pharmaceutical Sciences," 1990, 18th ed., Mack Publishing Co., Easton, PA. Suitable routes may include oral, rectal, transdermal, vaginal, transmucosal, or intestinal  
5 administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, just to name a few.

10 For injection, the substances of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants  
15 appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the  
20 active ingredients are contained in an amount effective to achieve its intended purpose. Determination of an effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable excipients and auxiliaries which facilitate processing of the active compounds into  
5 preparations which can be used pharmaceutically.

The present invention in one aspect provides for a method of inhibiting or reducing tissue injury attendant to angioplasty through the introduction of a therapeutic mutant gene capable of blocking a Ras signal  
10 transduction pathway. In one embodiment, the method comprises inducing one or more therapeutic genes capable of blocking a Ras signal transduction pathway into a patient having undergone an angioplasty procedure.

The invention further contemplates the  
15 treatment of other vascular disorders related to mechanical injury/fluid shear stress. These disorders include hypertension, arteriosclerosis, atherosclerosis, and reperfusion injury (Shyy and Chien, Cur Opin Cell Biol, 9:707-713; 1997).

20 The following non-limiting examples are included to demonstrate preferred embodiments of the invention. Those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed  
25 and still obtain a like or similar result without departing from the spirit and scope of the invention.

ExamplesEXAMPLE 1 - p21<sup>ras</sup> ACTIVATED BY FLUID SHEARING IN BAEC

5

p21<sup>ras</sup> is activated by fluid shearing in bovine aortic endothelial cells (BAEC). To demonstrate that fluid shearing leads to an activation of p21<sup>ras</sup> in ECs, i.e., an increased ratio of p21<sup>ras</sup>GTP to p21<sup>ras</sup>GDP, 10 confluent monolayers of the <sup>32</sup>P-labeled BAEC were subjected to a fluid shearing of 12 dynes/cm<sup>2</sup> for various time periods using the following method.

Bovine aortic endothelial cells prior to passage 10 were used in all the studies. The cells were 15 maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. All cell cultures were kept in a humidified 5% CO<sub>2</sub>-95% air incubator at 37°C. BAEC were cultured on 38 mm x 76 mm slides to confluence, and the slides were then assembled 20 into a rectangular flow channel with a height of 270 μm, through which the medium flows. The system was tightly sealed by using a silicon gasket and a vacuum line. A surface area of 14 cm<sup>2</sup> on the BAEC-seeded slide, confined by the gasket, was exposed to the applied fluid shearing, 25 which was generated by circulating the tissue culture medium through a hydrostatic pump connected to upper and



lower reservoirs. Frangos, et al., Science, 227:1477-1479, 1985. The pH of the system was kept constant by gassing with 95% Air-5% CO<sub>2</sub>, and the temperature was maintained at 37°C by immersing the flow system in a water bath. The shear stress, determined by the flow rate perfusing the channel and the channel thickness, was 12 dynes/cm<sup>2</sup> (1 dyne = 10<sup>-5</sup> Newton) which is relevant to the physiological range in the human major arteries and has been found to induce the expression of many IE genes in vitro. Hsieh, et al., J. Cell Physiol, 154:143-151, 1993; Sakaue, et al., Mol Cell. Biol., 15:379-388, 1995. The duration of the applied fluid shearing was 8 hr in the gene regulation experiments and varied from 1 to 60 min in the signal transduction experiments. Static control experiments were performed on BAEC on slides not exposed to fluid shearing.

The cells were lysed and P21<sup>ras</sup> guanine nucleotide binding assays were performed according to the procedures described previously by Downward et al. with minor modifications. Nature, 346:719-723, 1990. BAEC cultured on a glass slide were labeled with 0.5 mCi/ml [<sup>32</sup>P]orthophosphate (ICN Radiochemicals) for 6 hr in a phosphate-free medium. After labeling, the cells were subjected to fluid shearing or kept as static controls. Cell extracts were then prepared afterwards by lysing the BAEC in a buffer containing 50 mM Tris-HCl, pH 7.5, 0.5%-

NP-40, 0.15 M NaCl, 0.1 mM  $\text{Na}_3\text{VO}_4$ , 20 mM  $\text{MgCl}_2$ , 0.5% Triton X-100, 1  $\mu\text{g/ml}$  leupeptin, 1 mM PMSF, 2 mM DTT, and 2 mM  $\beta$ -glycerolphosphate. Ras proteins were immunoprecipitated with rat anti-p21<sup>ras</sup> mAb (Santa Cruz Biotech). The bound guanine nucleotides were separated from the precipitated protein complexes by using a buffer containing 20 mM Tris-HCl, pH 7.5, 20 mM EDTA, 2 mM DTT, 2% SDS, and 2 mM GTP. The eluted nucleotides were separated by thin layer chromatography using PEI-cellulose plates with 0.75 M  $\text{K}_2\text{HPO}_4$ , pH 3.4. The GDP and GTP contents were assessed by autoradiography.

In the static controls, p21<sup>ras</sup> was exclusively in its GDP-bound inactive form (Fig. 2). After shearing for 1 min., the ratio of p21<sup>ras</sup>GTP/p21<sup>ras</sup>GDP increased markedly. Densitometric analysis indicated that  $17 \pm 4\%$  of all the guanine nucleotides bound to p21<sup>ras</sup> was GTP bound. This GTP-bound active form gradually returned to the GDP-bound form afterwards. By 5 min after the beginning of shearing, the GTP bound form decreased to  $7 \pm 3\%$ . By 10 min, all p21<sup>ras</sup> became inactive, as in the static controls. Thus, fluid shearing, like other extracellular stimuli such as mitogens, cytokines, osmotic shock, and UV irradiation, induces a transient activation of p21<sup>ras</sup>.

EXAMPLE 2 - THE SHEAR-ACTIVATED AP-1/TRE IS MEDIATED  
THROUGH p21<sup>ras</sup>

A functional p21<sup>ras</sup> is required for the AP-  
5 1/TRE-mediated gene expression in response to fluid  
shearing. By using transient transfection assays, the  
present inventors show that the luciferase reporter  
driven by TRE (i.e., 4xTRE-P1-Luc and MCP1-Luc-540) can  
be induced by fluid shearing. Transactivation assays  
10 using c-Jun or c-Jun/c-Fos expression plasmids also  
induced these TRE-driven constructs indicating that the  
transcription factor that mediates such activation is AP-  
1. Shyy, et al., Proc. Natl. Acad. Sci. USA,  
91:4678-4682, 1994. To investigate whether Ras is  
15 upstream to the shear-induced activation of AP-1/TRE, we  
examined the effects of RasN17 on the induction of 4xTRE-  
P1-Luc and MCP1-Luc-540. RasN17 is a signaling  
incompetent mutant of Ras in which Ser-17 in the wild-  
type has been replaced by Asn, so that the affinity to  
20 GTP is dramatically reduced. Feig, et al., Mol. Cell.  
Biol., 8:3235-3242, 1988.

Expression plasmids encoding wild-type, or  
asignaling incompetent mutant of p21<sup>ras</sup> (RasN17) were co-  
transfected with either 4xTRE-P1-Luc or MCP1-Luc-540 into  
25 BAEC at 70% confluence by using standard transient  
transfection protocols. 4xTRE-P1-Luc is a construct in

which the rat prolactin promoter conjugated to luciferase reporter is driven by four copies of the TRE consensus sequence, and MCP1-Luc-540 is a construct in which the luciferase reporter is driven by the 540-bp MCP-1 promoter. Shyy, et al., Proc. Natl. Acad. Sci. USA, 91:4678-4682, 1994. The pSV- $\beta$ -galactosidase plasmid, which contains a  $\beta$ -galactosidase ( $\beta$ -gal) gene driven by SV40 promoter and enhancer, was also included in the co-transfection to monitor the transfection efficiency.

After incubation for 6 hr, the cells were washed with PBS and incubated with fresh media for another 24-48 hr to reach confluence. The cells in the tissue culture flasks were then seeded on glass slides and used either for fluid shearing experiments or as static controls. The luciferase reporter activities of the various experiments normalized for transfection efficiency were used to assess the suppressing effects of the various negative mutants on the shear-induced transcription activation mediated by AP-1/TRE.

As shown in Fig. 3A and Fig. 3B, in the plasmid control experiments, fluid shearing at 12 dynes/cm<sup>2</sup> for 8 hr. caused 23 and 6.5 folds of induction (the luciferase activities in the sheared cells compared to those in the static controls) for 4xTRE-P1-Luc and MCPI-Luc-540, respectively.

In a separate experiment BAECs were transfected with an expression plasmid encoding RasL61, the active form of p21<sup>ras</sup> in which the Gln-61 in the wild-type has been replaced by Leu. The expression of RasL61 increased the basal level expression for both 4xTRE-P1-Luc and MCPI-Luc-540. However, the induction by fluid shearing was not affected. In contrast, the co-transfection of RasN17 with either 4xTRE-P1-Luc or MCPI-Luc-40 into BAEC significantly decreased the shear-induced luciferase reporter activities. These results, together with those described in Example 1 (Fig 2), suggest that a functional p21<sup>ras</sup> is required for the AP-1/TRE-mediated gene expression in response to fluid shearing.

15

EXAMPLE 3 - FLUID SHEARING ACTIVATES JNK TO A GREATER  
EXTENT THAN ERK

This example demonstrates that fluid shear stress preferentially activates the Jnk signaling pathway in comparison to the Erk signaling pathway. ECs were exposed to shear stress and the ability of Jnk and Erk to phosphorylate a substrate was measured. Jnk activity was 10.5 fold higher than that measured in static cells after 30 min. whereas Erk activity was only 1.8 fold higher at its peak.

25

It has been shown previously that phosphorylation increases on Erks 1 & 2 upon fluid shearing of Ecs (Shyy, et al., Proc. Natl. Acad. Sci. USA, 91:4678-4682, 1994). To investigate whether the phosphorylation of ERK  
5 led to an increase in kinase activity, BAEC were subjected to shearing at 12 dynes/cm<sup>2</sup> for various lengths of time. After shearing, ERK was immunoprecipitated and the following kinase activity assay was performed. Five micrograms of anti MAPK/protein A-Agarose (UBS), in a  
10 buffer containing 20 mM HEPES (pH 7.7), 75 mM NaCl, 2.5 mM-MgCl<sub>2</sub>, 0.1 mM EDTA, 0.05% Triton X-100, 0.5 mM DTT, 20 mM  $\beta$ -glycerolphosphate, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 2  $\mu$ g/ml leupeptin, and 100  $\mu$ g/ml PMSF were added to the cell lysate to immunoprecipitate ERK. The suspension was  
15 mixed in 4°C for 4 hr and centrifuged. The pelleted beads were washed in phosphate buffered saline containing 0.1% Triton X-100, followed by resuspension in 30  $\mu$ l of a kinase buffer which contained 20 mM HEPES (pH 7.6), 20 mM MgCl<sub>2</sub>, 20 mM  $\beta$ -glycerolphosphate, 20 mM p-nitrophenyl  
20 phosphate, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 2 mM DTT, 20  $\mu$ M ATP, 5  $\mu$ g myelin basic protein (MBP), and 5  $\mu$ Ci [ $\gamma$  <sup>32</sup>P]ATP. After incubation at 30°C for 20 min, the kinase reaction was terminated by washing with HEPES binding buffer. The phosphorylated proteins were eluted in 30  $\mu$ l of 2x

Laemelli sample buffer and resolved on 10% SDS-polyacrylamide gel, followed by autoradiography.

The procedures for measuring JNK activity were the same as for ERK, except that 1  $\mu$ g agarose-bound glutathione-S-transferase (GST)-c-Jun(1-223) (Hibi, et al, Genes & Dev 7:2135-2148, 1993) and 5 $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP were added directly to the cell lysate for kinase reaction. A plasmid encoding hemeagglutinin (HA)-JNK was transfected into BAEC. This exogenous epitope-tagged JNK was immunoprecipitated with a mouse anti-HA mAb (Boehringer Mannheim). Densitometric analysis indicated that the peak fold of induction (compared to the static control) was 1.8x at 10 min, and 10.5x at 30 min.

Such a mechanical stimulation induced a rapid activation of ERK to cause MBP phosphorylation, which peaked at 10 min and decreased afterwards. Densitometric analysis indicated that the peak activity was 1.8 fold of that in the static controls. To investigate whether fluid shearing activates JNK as it does for ERK, agarose-bound GST-c-Jun, a fusion protein containing GST and the N-terminal moiety (1-223) of c-Jun, and [ $\gamma$ -<sup>32</sup>P]ATP were added to the cell lysate for JNK in BAEC to cause c-Jun phosphorylation, which peaked at 30 min and decreased afterwards. After the cells had been exposed to the shearing for 60 min, the JNK activities returned to a level lower than that in the static controls. The JNK

activity peaked, at 30 min and decreased afterwards. After the cells had been exposed to the shearing for 60 min, the JNK activities returned to a level lower than that in the static controls. The peak JNK activity, at 5 30 min determined by densitometry, was 10.5 fold of that in the static cells. Kinase assays on static cells incubated with fresh media or with conditioned media collected from sheared cells showed no difference in kinase activities from the static controls. These 10 results indicate that the activation of cytoplasmic kinases in the sheared cells was attributable to the action of mechanical force rather than by the media supplements or by the metabolites released from the cells during shearing. Furthermore, fluid shearing of BAEC 15 activates JNK to a greater extent and for a longer duration than ERK.

EXAMPLE 4 - p21<sup>ras</sup> AND MEKK ARE UPSTREAM TO THE SHEAR-  
ACTIVATED JNK

20

Fluid shearing activates a Ras - MEKK - JNK pathway in the vascular ECs. Ten micrograms of expression plasmid encoding HA-JNK was co-transfected with 10 µg of either SRα empty plasmids, RasN17, or 25 MEKK(K-M) (in which the Lys-432 had been replaced by a Met; Minden, et al., Science, 266:1719-1723, 1994) into



BAEC in a T-75 tissue culture flask. The transfected cells on culture slides were either kept as static controls or subjected to a fluid shearing of 12 dynes/cm<sup>2</sup> for 30 min. After shearing, HA-JNK was

5 immunoprecipitated with anti-HA mAb and subjected to kinase assays using GST-c-Jun and [ $\gamma$ -<sup>32</sup>P]ATP as substrates. Kinase assays indicate that the exogenous epitope-tagged HA-JNK was also activated by fluid shearing in the transfected BAEC. The immunoprecipitated

10 products were subject to gel electrophoresis, which indicated that RasN17 and MEKK(K-M) inhibited the shear-induced phosphorylation of GST-c-Jun. The cellular proteins were immunoblotted with anti-HA mAb following gel electrophoresis. The equal intensities of the bands

15 recognized by ECL detection reagents indicated that the amounts of the expressed HA-JNK were the same in different samples.

It has previously been shown that the expression of Ras activates MEKK and that the induction

20 of MEKK stimulates JNK. Lange-Carter and Johnson, Science 265:1458-1461, 1994; Yan, et al, Nature 372:798-800, 1994 These results indicate that fluid shearing activates a Ras - MEKK - JNK pathway in the vascular ECs.

25 EXAMPLE 5 - NEGATIVE MUTANTS OF MEKK AND JNK BLOCK THE  
SHEAR-ACTIVATED AP-1/TRE

This example shows that signal transduction incompetent MEKK and JNK can abrogate Jnk pathway signaling *in vitro*.

- 5           MEKK and JNK are upstream to the AP-1/TRE mediated gene expression in response to shear stress. To examine whether MEKK mediates the shear-induced reporter driven by AP-1/TRE, co-transfection of MEKK (K-M) with either 4xTRE-P1-Luc or MCP-540 was done. In addition,
- 10 JNK (K-R), a kinase deficient JNK1, in which the Lys-52 in the wild-type was replaced by an Arg, was constructed. To construct JNK(K-R), JNK 1 was mutated in pBluescript (Stratagene) by PCR to introduce a NcoI site at its first ATG codon and a point mutation at codon 55 which replaced
- 15 the Lys-55 with an Arg. The mutations were confirmed by DNA sequencing. The mutated JNK was then subcloned into expression vector SR $\alpha$ 3HA (Deng and Karin, *Nature, London*. 371:171-175, 1994) at NcoI and BgIII sites to create JNK(K-R).
- 20           If the Ras- MEKK - JNK pathway is upstream to the AP-1/TRE, the use of either MEKK(K-M) or JNK(K-R) to block the functions of the wild-types should attenuate the shear-induced AP-1/TRE transcription. Fig. 4A indicates that the co-transfection of expression plasmids
- 25 encoding MEKK(K-M) or JNK(K-R) did attenuate the shear-induced 4xTRE-P1-Luc from 21.5 folds to 12.5 and 4 folds,

respectively. Co-transfection of these catalytically inactive mutants with MCPI-Luc-540 also reduced the shear induced luciferase reporter activities (Fig. 4B). In contrast, co-transfection of the expression plasmids encoding the wild-type MEKK or JNK did not affect the shear-induced 4XTRE-P1-Luc or MCPI-Luc-540. Thus, MEKK and JNK are upstream to the AP-1/TRE mediated gene expression in response to shear stress.

10 EXAMPLE 6 - RAF-301, ERK(52R), AND ERK(71r) HAVE LITTLE EFFECT ON THE SHEAR-ACTIVATED AP-1/TRE

This example demonstrates that signal transduction incompetent proteins from the Erk pathway do not block shear induced transcription mediated by AP-1/TRE, in contrast to the result seen in Example 5.

The Ras - Raf-1 - ERK pathway is not essential for the shear-induced activation of AP-1/TRE. In response to stimulation by growth factors or phorbol ester, the Ras-ERK pathway is activated, leading to the activation of AP-1/TRE. DeVries-Smits, et al, Nature, 357:602-604, 1992; Thomas, et al, Cell 68:1031-1040, 1992; Frost, et al., Proc. Natl Acad. Sci., USA, 91:3844-3848, 1994 Raf-1 contributes directly to ERK activation in this pathway, but not to JNK activation. Mechanical shearing has a less potent effect on ERK than

on JNK (Example 3) which seems to indicate that the Ras - Raf -1 - ERK pathway is less important for downstream gene expression. To test the role played by the Ras - Raf-1 - ERK pathway in the shear-induced activation of AP-1/TRE, we used the signal transduction incompetent mutants of Raf-1 and ERK to block this pathway and examined the AP-1/TRE-mediated reporter activities in response to mechanical stimulation.

Raf301 is a signal transduction incompetent mutant of Raf-1 in which the Lys-375 in the wild-type has been replaced by Trp. Kolch, et al., *Nature*, 349:426-428, 1991. ERK(K71R) and ERK(K52R) are the dominant mutants of ERK1 and ERK2, in which the respective Lys71 and Lys-52 in the wild types has been replaced by Arg. Rovvins, et al, *JBC* 268:5097-5106, 1993. Fig. 5 shows that co-transfection of Raf301 had little effect on the shear-induced 4xTRE-P1-Luc reporter activity. Similarly, neither ERK(71R), ERK(52R), nor a combination of these two ERK signal transduction incompetent mutants attenuated the reporter activity in response to fluid shearing. Experiments using MCP1-Luc-540 also showed that none of these negative mutants was able to affect the shear-induced luciferase. Thus, the Ras - Raf-1 - ERK pathway is not essential for the shear-induced activation of AP-1/TRE.

EXAMPLE 7 - FLUID SHEARING INCREASES c-JUN  
TRANSCRIPTIONAL ACTIVITY

- 5                    Fluid-shearing induced activation of AP-1, is  
at least in part, due to an increased c-Jun  
transcriptional activity, which is in turn activated  
through the phosphorylation of Ser-63 and -73 by the Ras  
- MEKK - JNK pathway. The induction of 4xTRE-P1-Luc and  
10    MCPI-Luc-540 by fluid shearing results from an up-  
regulated c-Jun, which is activated by the Ras - MEKK -  
JNK pathway. To test whether fluid shearing increases  
the transcriptional activity of c-Jun, plasmid Gal4-c-Jun  
(1-223) encoding the fusion protein of Gal4 DNA binding  
15    domain and c-Jun activation domain (1-223) (Minden, et  
al., Mol. Cell. Biol., 14:6683-6688, 1994) were co-  
transfected with 4xGal-Luc, a chimeric construct  
consisting of the Gal4-binding sequence and the  
luciferase reporter, into BAEC.
- 20                    Compared to the static controls, fluid shearing  
increased the luciferase activity by more than 4 folds in  
the sheared cells (Figs. 6A, 6B), indicating an increased  
c-Jun transcriptional activity. In contrast, the plasmid  
encoding the mutated Gal4-Jun, in which the  
25    phosphorylation sites Ser-63 and -73 had been replaced by  
Ala, showed a marked reduction in response to fluid

shearing. Furthermore, co-expression of RasN17 or MEKK (K-M) also attenuated such shear-induced transcriptional activity. Thus, the fluid-shearing induced activation of AP-1 is at least in part due to an increased c-Jun transcriptional activity, which is in turn activated through the phosphorylation of Ser-63 and -73 by the Ras - MEKK - JNK pathway.

EXAMPLE 8 - ΔmSOS1 ATTENUATES THE SHEAR-INDUCED c-JUN  
10 TRANSCRIPTIONAL ACTIVITY

ΔmSOS1 attenuates the shear-induced c-Jun transcriptional activity. Son of sevenless (Sos) is a guanine nucleotide exchange factor that activates p21<sup>ras</sup> by converting the GDP-bound inactive state to the GTP-bound active state. Chardin, et al., Science, 260:1338-1343, 1988; Egan, et al., Nature, 363:45-51, 1993.

To explore whether Sos is a upstream molecule regulating the shear-activated Ras signaling, expression plasmids encoding ΔmSOS1, a signal transduction incompetent mutant of mouse SOS 1 in which the guanine nucleotide exchange domain has been deleted, were co-transfected with Gal4-c-Jun(1-223) and 4xGal-Luc into BAEC. Sakaue, et al., Mol. Cell. Biol., 15:379-388, 1995. The transfected cells were then subjected to fluid shearing/luciferase assays. As shown in Fig. 6B, ΔmSOS1

attenuates the shear-induced c-Jun transcriptional activity. The inducibility by fluid shearing in cells transfected with mSOS1, the wild-type mouse SOS1, was comparable to that in cells transfected with empty  
5 vectors.

EXAMPLE 9 - RASN17 INHIBITS THE AP-1/TRE-MEDIATED  
TRANSACTIVATION IN RESPONSE TO SERUM OR PDGF

10 AP-1/TRE-mediated gene expression (e.g., MCP-1) induced by mitogenic stimuli in vascular endothelium can be blocked by RasN17. The cloned RasN17 plus adenovirus shuttle vector (see Example 10) and a luciferase reporter driven by PTA-responsive elements (4xTRE-P1-Luc) were co-  
15 transfected into BAEC. The transfected cells in serum free medium were treated with either 15% serum or 10 ng/ml PDGF for 24 h after which luciferase activity was measured as previously described. Figure 7 shows that the induction of luciferase activity by serum or PDGF in  
20 the control cells is significantly attenuated by the co-transfection of RasN17. These results indicate that the AP-1/TRE-mediated gene expression (e.g., MCP-1) induced by mitogenic stimuli in vascular endothelium can be blocked by RasN17.

EXAMPLE 10 - CONSTRUCTING AND CONFIRMING THE RASN17 cDNA  
SEQUENCE

Construction of recombinant adenoviruses

5           A recombinant adenovirus, AdRasN17, was assembled by transfecting 293 embryonic kidney cell line (ATCC CRL 1573) with pACCMVpLpA (8.8 Kb) (Gomez-Foix, et al, JBC 267:25129-25134, 1992), a shuttle vector containing RasN17 cDNA, and pJM17, a vector carrying a  
10 dl309 adenovirus 5 genome (Ad5) (McGrory, et al, Virology 163:614-617, 1988). The pJM17 contains the full length Ad5 genome (36 kb) with the interruption of a 4.3 kb unrelated DNA fragment at position map units, thereby exceeding the adenoviral packaging limit. After the  
15 homologous recombination between PACCMBpLpA and pJM17 in 293 cells, the adenovirus El region responsible for viral replication was substituted by the RasN17 expression cassette, resulting in replication-deficient viruses. Putative viral clones were plaque purified, propagated,  
20 isolated, and the titer was determined according to established procedures. A Southern blot was performed to verify the insertion of RasN17 DNA into viruses. The control adenoviral vector containing a LacZ gene expression cassette (AdLacZ) was constructed by similar  
25 procedures. AdLacZ was obtained from 293 cells co-transfected with pJM17 and pXCJL 1/CMV/nls-LacZ, a



derivative of pXCJL.1 that carries an expression cassette in which the human cytomegalovirus IE promoter encoding the SV40 large T-antigen nuclear-localization signal was fused to the E. coli LacZ reporter gene.

EXAMPLE 11 - ADENOVIRUS-MEDIATED GENE DELIVERY IS AN  
EFFICIENT METHOD FOR GENE TRANSFER TO PIG SMOOTH MUSCLE  
CELLS

5           The transfection efficiency of Ad-RasN17 was compared to that of a liposome transfection system. By introducing a reporter gene LacZ into porcine smooth muscle cells (PSMC) and staining with X-Gal, it was found that the transfection efficiency of DNA porcine smooth  
10 muscle cells by liposome is poor. Only approximately 5% of cells can be transfected. In order to test DNA transfection efficiency mediated by adenovirus, an adenovirus containing LacZ gene, Ad-LacZ, was used to determine the transfection efficiency. Ad-LacZ was  
15 transfected into PMSMC, with the result that the majority of the smooth muscle cells (>95%) were X-Gal staining positive, indicating the successful transfection of these cells by Ad-LacZ. Thus, adenoviral vector is superior to liposomes as a delivery system to transfer therapeutic  
20 genes into vascular cells.

EXAMPLE 12 - COCKTAIL OF RASN17 AND OTHER MOLECULES IN  
RAS PATHWAYS

25           An adenovirus-based system to deliver signal transduction incompetent mutants in the Ras-mediated

signaling pathway to vascular cells was developed in order to block the Ras pathway in a comprehensive manner so that the expression of genes involved in atherogenesis and restenosis can be effectively abolished. These  
5 molecules, which include, but are not limited to, signal transduction incompetent of FAK, c-src, Son of Sevenless (Sos), MEKK, JNKK, JNK, Raf and ERK, have previously been shown to attenuate the downstream gene expression.

10 EXAMPLE 13 - USE OF RASN17 TO ATTENUATE VASCULAR SMOOTH  
MUSCLE PROLIFERATION IN VITRO

Expression of RasN17 in SMC can significantly attenuate the growth of the cells. The present example  
15 is provided because the proliferate response of VSMC to high-serum or PDGF culture conditions in vitro resembles their hyperplastic response to balloon injury in vivo. 15% serum or 10 ng/ml PDGF was applied to the serum starved porcine VSMC after transfection with a  
20 replication-deficient virus carrying the RasN17 gene (Ad-RasN17). In controls, replication-deficient virus carrying the LacZ gene (Ad-LacZ) was used. The <sup>3</sup>H-Thymidine incorporation assay was performed to assess cell proliferation. In vitro, VSMC stimulated with serum  
25 and PDGF showed proliferate response in the Ad-LacZ

control group, but this was inhibited in the Ad-RasN17 transfected group.

Adenovirus-mediated transfection and  $^3\text{H}$ -Thymidine

5 incorporation assays

PSMCs were seeded on 96-well plates and grown to 50% confluence. The cells were then infected with the replication-deficient adenovirus AdlacZ or AdRasN17 in  $1 \times 10^6$ ,  $1 \times 10^7$ , or  $1 \times 10^8$  plaque-forming units per milliliter (pfu/ml). 24-hr after infection, the infected cells were starved in DMEM containing 0.5% serum for 24 hr followed by stimulation with 15% serum or 10 ng/ml PDGF. The cells were pulse-labeled for 4 hr in growth media containing 2.5  $\mu\text{Ci/ml}$  methyl- $^3\text{H}$  Thymidine (Amersham Life Science). The cells were trypsinized and collected on glass fiber filter papers. The filter papers were collected in polypropylene vials, mixed with 5 ml scintillate per sample for 12 hr, and counted in a beta scintillation counter.

20

RasN17 inhibits the proliferation of PSMCs in response to mitogens

Serum-starved PSMCs infected with a replication-deficient virus carrying the RasN17 (Ad-RasN17) or the control replication-deficient virus carrying the lacZ gene (Ad-lacZ) were subjected to

25

stimulation with 15% serum or 10 ng/ml PDGF. As shown in FIGS 8A and 8B, <sup>3</sup>H-Thymidine incorporation assay showed that cell proliferation increased by 48 times in the serum-stimulated PSMCs and 15 times in the PDGF-stimulated PSMCs infected with Ad-lacZ (10<sup>8</sup> pfu/ml) as controls. In contrast, the serum- and PDGF-stimulated proliferation was reduced drastically for PSMCs infected with Ad-RasN17 at 10<sup>8</sup> pfu/ml. These results indicate that expression of RasN17 in SMC can significantly attenuate the growth of the cells.

EXAMPLE 14 - USE OF RAS N17 GENE THERAPY TO PREVENT  
ARTERY RESTENOSIS IN VIVO

This example shows that the introduction of a gene encoding a signal transduction incompetent protein that blocks Jnk pathway signaling is a useful therapeutic *in vivo*.

Percutaneous transluminal coronary angioplasty (PTCA) has been extensively used as a clinical approach to treat coronary heart disease. However, restenosis occurs at the site of angioplasty in approximately one third of the patents within 6 months after PTCA. An important factor in restenosis is that the abrasive actions on the vessel wall during the PTCA procedure denude the endothelial cells and traumatize the vessel

media, leading to the inflammatory and proliferative responses of smooth muscle cells to cause restenosis.

Our in vitro studies on cultured pig smooth muscle cells showed that the introduction of RasN17, a signal transduction incompetent mutant of p21Ras, into those cells inhibited the expression of genes that lead to the cell proliferation response to several mitogens (e.g., PDGF) and hemodynamic force (e.g. shear stress). These led us to perform animal experiments to test the in vivo efficacy of negative mutants in the pathway in preventing restenosis after PTCA. The aim was to provide an effective method for reduction of the high incidence of restenosis after angioplasty and other surgical interventions in patients with coronary heart disease.

Our results show that the restenosis of rat common carotid arteries after balloon injury is inhibited by local treatment with recombinant adenovirus-carrying RasN17.

#### Animal experiments

The rats were anesthetized with Ketamine (100 mg/kg body weight, i.p.) and Xylazine (10 mg/kg body weight, i.p.). Under sterile conditions, a neck incision was made and the left carotid artery was exposed. The common carotid artery (CCA) was clamped at 2 cm proximal to the bifurcation, and the internal carotid artery (ICA)

was also clamped at its proximal position. The external carotid artery (ECA) was ligated at 1 cm distal to the bifurcation and a small arteriotomy was made just proximal to the ligation site for the insertion of a  
5 balloon catheter into the CCA. Vascular injury was achieved by inflating the balloon at 1.8 ATM, and then sliding the balloon catheter back and forth three times. After the deflation of the balloon and the withdrawal of the catheter, a vascular clamp was placed at 1 cm  
10 proximal to the bifurcation, and adenovirus (50 $\mu$ l,  $10^9$  pfu/ml) was injected through the arteriotomy into the segment distal to the clamp. After 15-min incubation, the virus was removed, and the clamp was removed to restore blood flow. The neck incision was closed, and  
15 the rat was allowed to recover with normal husbandry procedures for two weeks. Then, the animal was sacrificed with an overdose of anesthesia and infused with PBS and 4% para-formaldehyde phosphate buffer at 100 mm Hg for 10-15 min. CCA was removed and fixed overnight  
20 for histological staining with hemtaoxylin-eosin.

Ad-RasN17 inhibits restenosis in the rat model after  
balloon injury

The balloon procedure in the common carotid  
25 arteries of ten rats was performed with Ad-RasN17 or Ad-LacZ injected into the distal segment of the vessel. Ad-

RasN17 was injected in five of the animals as the experimental group, and Ad-LacZ in the other five as controls. Histological examination was made to determine the intimal/media cross-sectional area ratio (I/M ratio) for the assessment of restenosis. Cross sections of the CCA indicated that in the control group, two of the five animals developed restenosis in both the proximal and distal segments, and the I/M ratio was  $1.99 \pm 0.23$ . In the experimental group, restenosis developed in two of the five animals, but only in the proximal, untreated segment of their vessels; the I/M ratio there was not significantly different from that in the control group. The distal segments of these experimental animals, where Ad-RasN17 injection was made, showed little evidence of restenosis, and the I/M ratio ( $0.95 \pm 1.15$ ) was markedly lower than that in the proximal segment of the same animals or the segments in the control group. These results indicate that Ad-RasN17 is a potential therapeutic gene for the prevention of post-angioplasty restenosis.

As shown in Fig. 9 the administration of RasN17 into rat common carotid arteries markedly reduced the restenosis of the vessel after balloon injury in vivo. Therefore, Ad-RasN17 is a potential therapeutic gene for the prevention of post-angioplasty restenosis. The same experiment was conducted in



vivo in pigs. This experiment showed that Ad-RasN17 also inhibits restenosis in pigs.

EXAMPLE 15 - USE OF RASN17 DERIVATIVES AS THERAPEUTIC

5 GENE IN THE PREVENTION OF RESTENOSIS

A partial sequence of RasN17 containing the mutated GTP binding site is postulated to be sufficient to be functional in inhibiting the proliferation of  
10 smooth muscle cells after PTCA. This truncated gene contains the 80 amino acids at the NH<sub>2</sub>-terminal of RasN17 with the sequence shown below:

MTEYKLVVVGAGGVGKNALTIQLIQNHVFVDEYDPTIEDSYRKQVVIDGETCLLDILD  
TAGLEEYSAMRDQSMRTGEGFLC (SEQ ID NO: 1) (amino acids 1-80

15 Ras)

Several mutants, e.g., RasA17, RasG17, and RasK17 in which Ser-17 in the wild-type is replaced by Ala, Gly, or Lys, respectively, will be constructed (see table below for coding  
20 sequence). These mutants will be tested for their therapeutic effects in prevention of restenosis after PTCA.

<u>Amino Acid</u>	<u>Nucleotide Sequence</u>
Ala(A)	GCT; GCC; CGA; GCG
Gly(G)	GGT; GGC; GGA; GGG
25 Lys(K)	AAG; AAA

EXAMPLE 16 - NEGATIVE MUTANTS OF SRC ATTENUATE THE SHEAR  
STRESS ACTIVATION OF JNK

p60src is upstream to p21ras in the shear stress activation of JNK in BAEC. To investigate whether the shear-induced p60src can be upstream to JNK, BAECs were co-transfected with the 3  $\mu$ g of epitope-tagged HA-JNK1 together with 6  $\mu$ g of either an expression plasmid encoding v-src(K295R), a kinase-defective mutant of v-src (Fig. 10A, lanes 3 and 4), or an empty vector as a control (Fig. 10A, lanes 1 and 2). As in Example 1, transfected cells were subject to a shear stress of 12 dyn/cm<sup>2</sup> for 30min. or kept as a static control. Kinase assays, as in Example 1, were performed and demonstrated that shearing the control cells (transfected with empty plasmid) for 30 min increased the kinase activity of HA-JNK1 which was demonstrated by the phosphorylation of GST-c-Jun(1-79) (lane 2 vs. lane 1 in Fig. 10A). In contrast, co-transfection of v-src(K295R) markedly attenuated the shear-induced JNK activity (lane 4 vs. lane 2 in Fig. 10A). Densitometry analysis showed that shear stress caused a 6.0-fold increase in the kinase activity of HA-JNK1 in control cells and that this was reduced to a 1.7-fold in cells co-transfected with v-src(K295R).

In a separate set of experiments (Fig. 10B) shear stress increased the kinase activity of HA-JNK1 in c-src overexpressed cells (transfected with c-src(wt) encoding wild-type c-src). Co-transfection of 3 µg HA-JNK1 with 6 µg c-src(K295R), a kinase-defective mutant of p60src, resulted in attenuation of the shear stress activation of HA-JNK1. (lane 4 vs. lane 1 in Fig. 10B). Examined by immunoblotting, the levels of the expressed HA-JNK1 in these BAEC were essentially the same, indicating that the expression of HA-JNK1 was not affected by co-transfection of the various plasmids.

To further demonstrate the requirement of p21ras in the induction of JNK by p60src, experiments were conducted to determine whether a constitutively activated p60src, i.e., c-src(F527) can activate JNK, and if it does, whether the activation can be blocked by RasN17, a signal transduction incompetent mutant of p21ras. Co-transfection of 6 µg c-src(F527) with 3 µg HA-JNK1 increased the kinase activity of HA-JNK1 (Fig. 10C, lane 2). Co-transfection of 12 µg RasN17 markedly reduced the c-src(F527)-induced HA-JNK1 kinase activity (Fig. 10C, lane 3). Co-transfection of 6 µg RasL61 (activated form of p21ras) also increased the HA-JNK1 activity (Fig. 10C, lane 4). but this could not be decreased by co-transfection of 12 µg v-src(K295R) (Fig. 10C, lane 5). These results in conjunction with those

shown in Fig. 10A indicate that p60src is upstream to p21ras in the shear stress activation of JNK in BAEC.

All of the compositions and methods disclosed  
5 and claimed herein can be made and executed without undue experimentation in light of the present disclosure.  
While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations  
10 may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically  
15 and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the  
20 invention as defined by the appended claims.

All of the compositions and methods disclosed  
and claimed herein can be made and executed without undue experimentation in light of the present disclosure.  
25 While the compositions and methods of this invention have been described in terms of preferred embodiments, it will

be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and

5 scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and

10 modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

## WHAT IS CLAIMED IS:

1. A method of inhibiting the fluid shear  
5 stress response of a vascular cell comprising inhibiting  
a Ras signal transduction pathway.
2. A method according to claim 1 wherein said  
Ras signal transduction pathway is a Jnk signal  
10 transduction pathway.
3. A method according to claim 2 wherein said  
Ras signal transduction pathway is inhibited by  
introducing a gene encoding a signaling incompetent Ras  
15 protein into the cell to be inhibited.
4. A method according to claim 3 wherein said  
signaling incompetent Ras protein comprises a single  
amino acid modification.  
20
5. A method according to claim 3 wherein said  
signaling incompetent Ras protein comprises multiple  
amino acid modifications.
- 25 6. A method according to claim 3 wherein said  
gene comprises an antisense Ras gene.

7. A method according to claim 3 wherein said gene encoding a signaling incompetent Ras protein is contained on a gene therapy expression vector.

5

8. A method according to claim 7 wherein said gene therapy expression vector is an adenovirus vector.

9. A method according to claim 3 additionally  
10 comprising introducing a second gene encoding a second signaling incompetent protein into the cell to be inhibited.

10. A method according to claim 9 wherein said  
15 second signaling incompetent protein is a signaling incompetent MekK protein, a signaling incompetent JnkK protein, a signaling incompetent Jnk protein, a signaling incompetent c-Jun protein or a signaling incompetent src protein.

20

11. A method according to claim 9 wherein the second signaling incompetent protein is a signaling incompetent Fak protein, a signaling incompetent IKK protein, a signaling incompetent Shc protein, a signaling  
25 incompetent Grb2 protein or a signaling incompetent Sos protein.

12. A method according to claim 2 wherein said Jnk signal transduction pathway is inhibited by introducing a gene encoding a signaling incompetent MekK protein, a signaling incompetent JnkK protein, a signaling incompetent Jnk protein, a signaling incompetent c-Jun protein, a signaling incompetent Ras protein, or a signaling incompetent src protein into the cell to be inhibited.

10

13. A method according to claim 12 wherein said signaling incompetent protein is a signaling incompetent MekK protein, a signaling incompetent Jnk protein, a signaling incompetent Ras protein, or a signaling incompetent src protein.

15

14. A method according to claim 13 wherein said signaling incompetent MekK protein is MEKK(K-M).

20

15. A method according to claim 13 wherein said signaling incompetent Jnk protein is JNK(K-R).

16. A method according to claim 13 wherein said signaling incompetent src protein is v-src (K-R).

25



17. A method according to claim 13 wherein said signaling incompetent Ras protein is RasN17.

18. A method according to claim 1 wherein the  
5 vascular cell is a vascular endothelial cell.

19. A method according to claim 1 wherein the vascular cell is a vascular smooth muscle cell.

10 20. A pharmaceutical composition used in treating disorders related to fluid shear stress response by inhibiting a Ras signal transduction pathway.

21. A pharmaceutical composition according to  
15 claim 20 wherein the Ras signal transduction pathway is a Jnk signal transduction pathway.

22. A pharmaceutical composition according to claim 20 comprising one or more genes encoding one or  
20 more signaling incompetent proteins.

23. A pharmaceutical composition according to claim 22 wherein said gene or genes encode a signaling incompetent MekK protein, a signaling incompetent JnkK  
25 protein, a signaling incompetent Jnk protein, a signaling

incompetent c-Jun protein, a signaling incompetent Ras protein or a signaling incompetent src protein.

24. A pharmaceutical composition according to  
5 claim 22 wherein said gene or genes encode a signaling  
incompetent Fak protein, a signaling incompetent IKK  
protein, a signaling incompetent Shc protein, a signaling  
incompetent Grb2 protein or a signaling incompetent Sos  
protein.

10

25. A pharmaceutical composition according to  
claim 23 wherein said gene or genes encode a signaling  
incompetent MekK protein, a signaling incompetent Jnk  
protein, a signaling incompetent Ras protein or a  
15 signaling incompetent src protein.

26. A pharmaceutical composition according to  
claim 25 wherein said signaling incompetent MekK protein  
is MEKK(K-M).

20

26. A pharmaceutical composition according to  
claim 24 wherein said signaling incompetent Jnk protein  
is JNK(K-R).

28. A pharmaceutical composition according to claim 25 wherein said signaling incompetent Ras protein is RasN17.

5 29. A pharmaceutical composition according to claim 25 wherein said signaling incompetent src protein is Src (K-R).

30. A pharmaceutical composition according to  
10 claim 22 wherein the composition comprises at least two genes encoding at least two signaling incompetent proteins.

31. A pharmaceutical composition according to  
15 claim 30 wherein the two signaling incompetent proteins are a signaling incompetent Ras protein and a signaling incompetent src protein.

32. A pharmaceutical composition according to  
20 claim 31 wherein the signaling incompetent Ras protein is RasN17 and the signaling incompetent src protein is src (K-R).

33. A pharmaceutical composition according to  
25 claim 20 comprising an antisense gene or a chemical inhibitor compound.

34. A pharmaceutical composition according to claim 22 wherein said gene encoding a signaling incompetent Ras protein is contained on a gene therapy  
5 expression vector.

35. A pharmaceutical composition according to claim 34 wherein said gene therapy expression vector is an adenovirus vector.

10 36. A method of treating a vascular disorder comprising inhibiting a Ras signal transduction pathway.

37. A method according to claim 36 wherein the vascular disorder comprises restenosis, atherosclerosis,  
15 or reperfusion injury.

38. A method according to claim 36 wherein the Ras signal transduction pathway is Jnk signal transduction pathway.

20

39. A method according to claim 38 wherein the vascular disorder comprises restenosis, atherosclerosis, or reperfusion injury.

40. The use of a gene encoding a signaling incompetent protein in the treatment of a vascular disorder.

5           41. The use according to claim 40 wherein the vascular disorder comprises restenosis, atherosclerosis or reperfusion injury.

          42. The use according to claim 40 wherein the  
10 signaling incompetent protein comprises a signaling incompetent MekK protein, a signaling incompetent JnkK protein, a signaling incompetent Jnk protein, a signaling incompetent c-Jun protein, a signaling incompetent Ras protein or a signaling incompetent src protein.

15

          43. The use according to claim 42 wherein the gene is contained on a gene therapy expression vector.

          44. The use according to claim 43 wherein the  
20 gene therapy expression vector is an adenovirus expression vector.

          45. A nucleic acid sequence comprising a gene encoding a signaling incompetent JNK protein or a  
25 signaling incompetent MEKK protein.

46. A nucleic acid sequence of claim 45 wherein the signaling incompetent JNK protein is JNK (K-R).

5 47. A nucleic acid sequence according to claim 46 wherein the signaling incompetent MEKK protein is MEKK (K-M).

48. A nucleic acid sequence comprising a gene  
10 therapy expression vector and a gene encoding a signaling incompetent MekK protein, a signaling incompetent JnkK protein, a signaling incompetent Jnk protein, a signaling incompetent c-Jun protein, a signaling incompetent Ras protein or a signaling incompetent src protein.

15

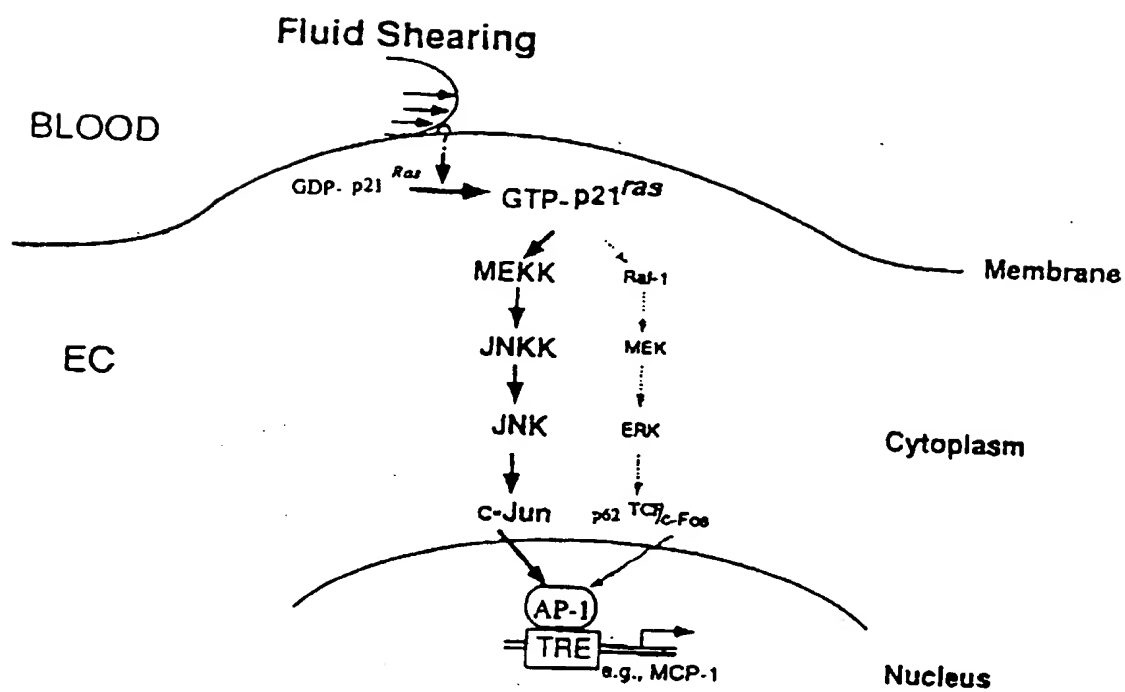
49. A nucleic acid sequence according to claim 48 wherein the gene therapy expression vector comprises a retrovirus vector, a vaccinia virus vector, an adenovirus vector, a herpes virus vector or a bovine papilloma virus  
20 vector.

50. A nucleic acid sequence for use in the method of claim 1 comprising a gene therapy expression vector and a gene encoding a signaling incompetent MekK  
25 protein, a signaling incompetent JnkK protein, a signaling incompetent Jnk protein, a signaling

incompetent c-Jun protein, a signaling incompetent Ras protein or a signaling incompetent src protein.

51. A nucleic acid sequence according to claim  
5 50 wherein the gene therapy expression vector comprises a retrovirus vector, a vaccinia virus vector, an adenovirus vector, a herpes virus vector or a bovine papilloma virus vector.

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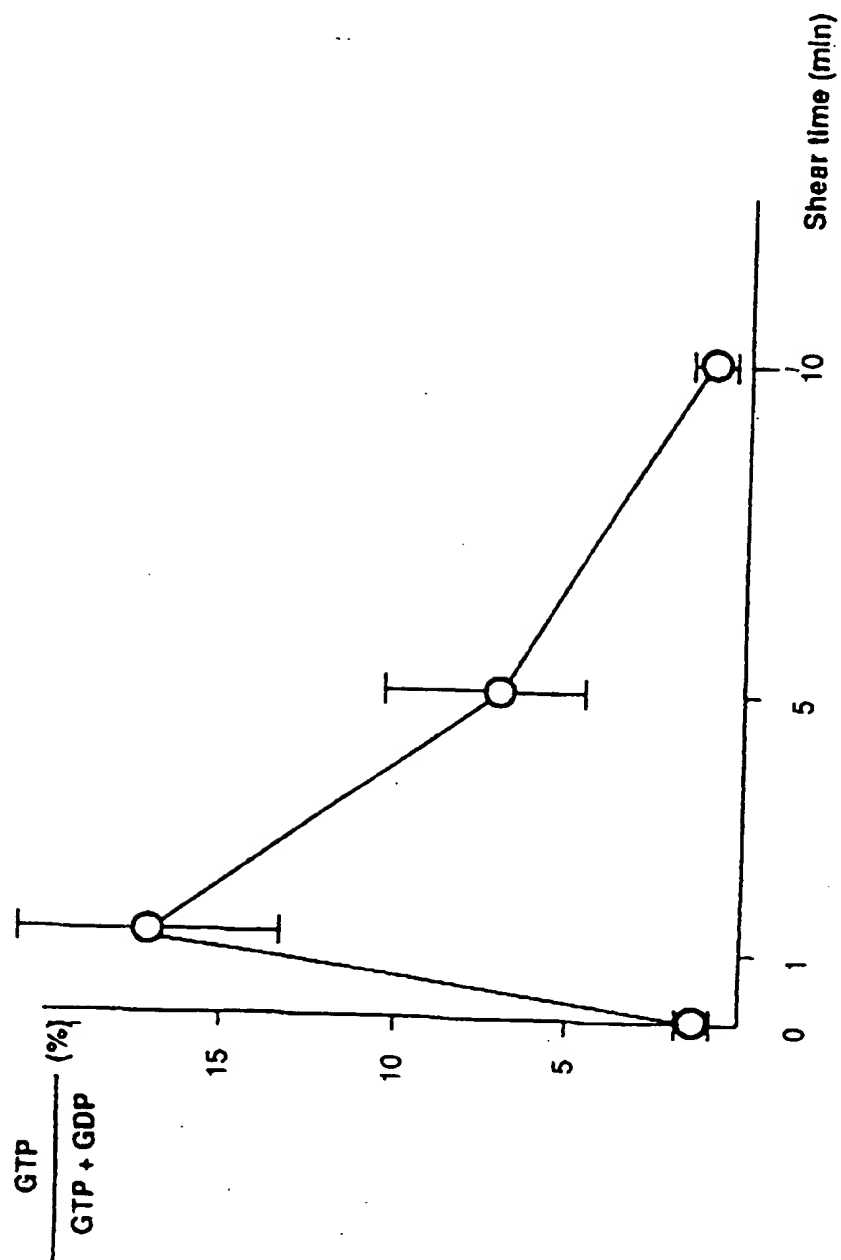
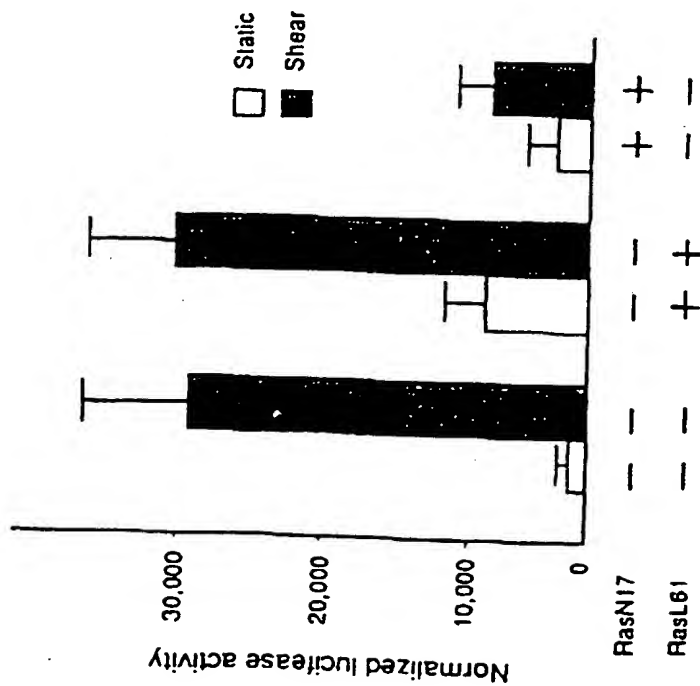


Fig. 2

Fig. 3

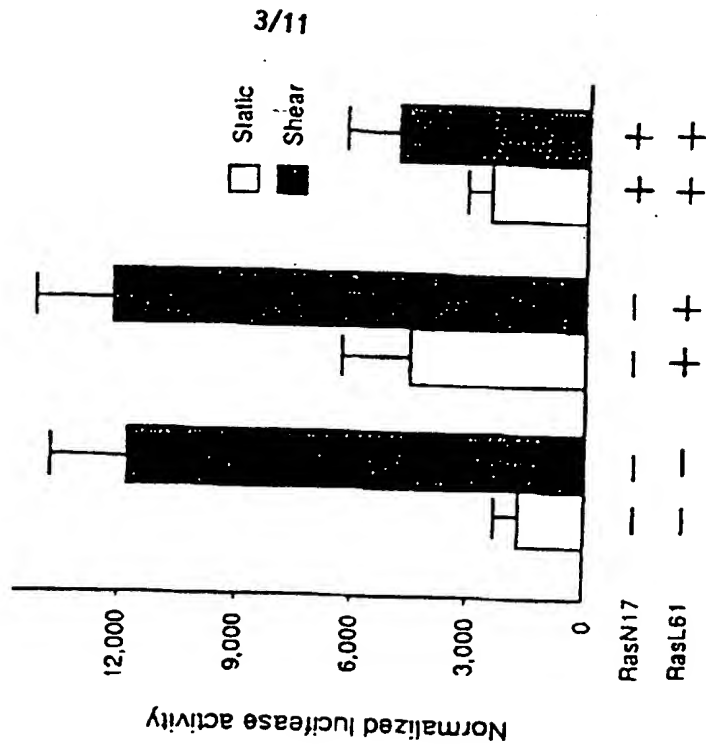
A.

4xTRE-PI-Luc



B.

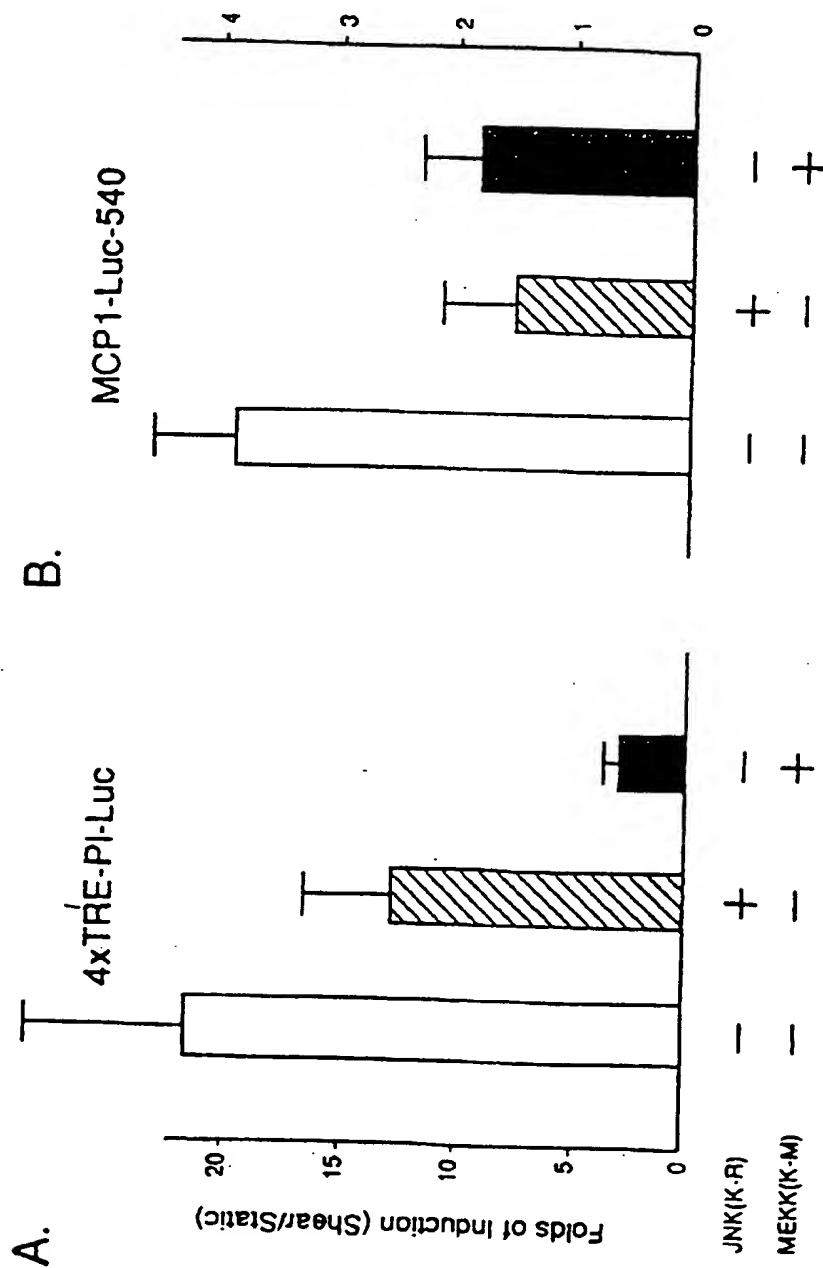
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Fig. 4



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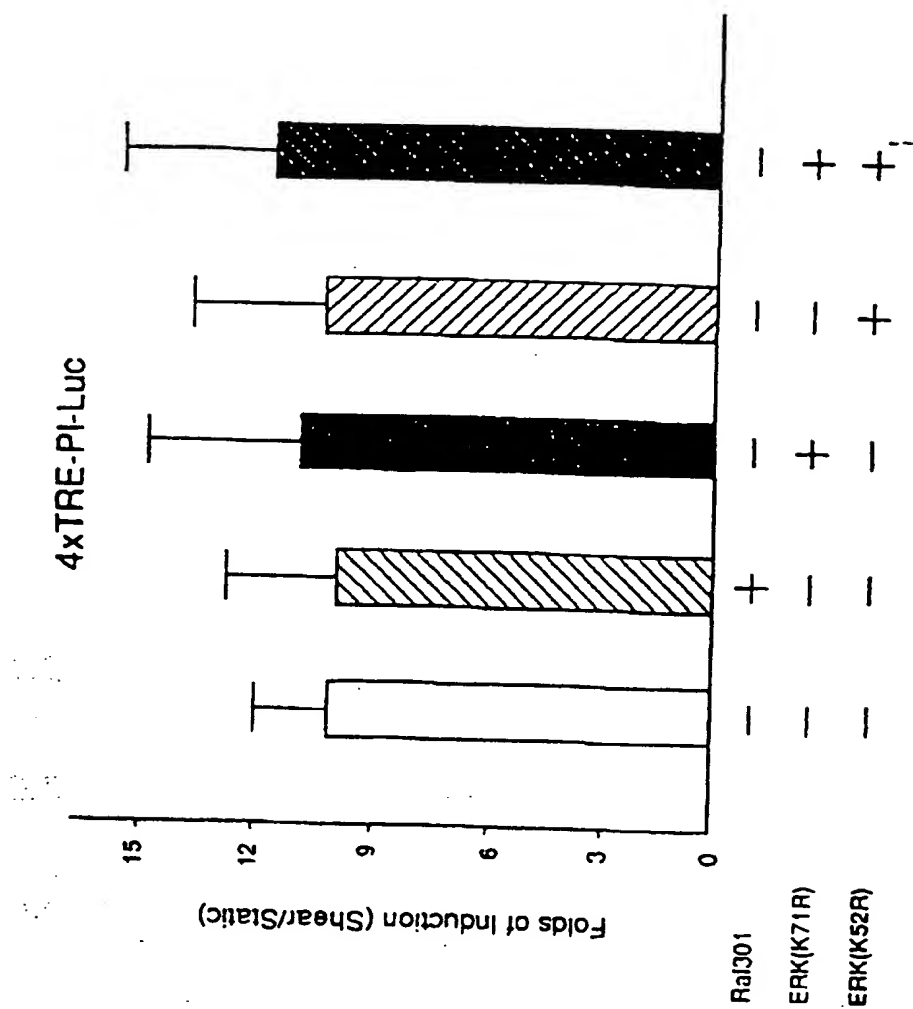
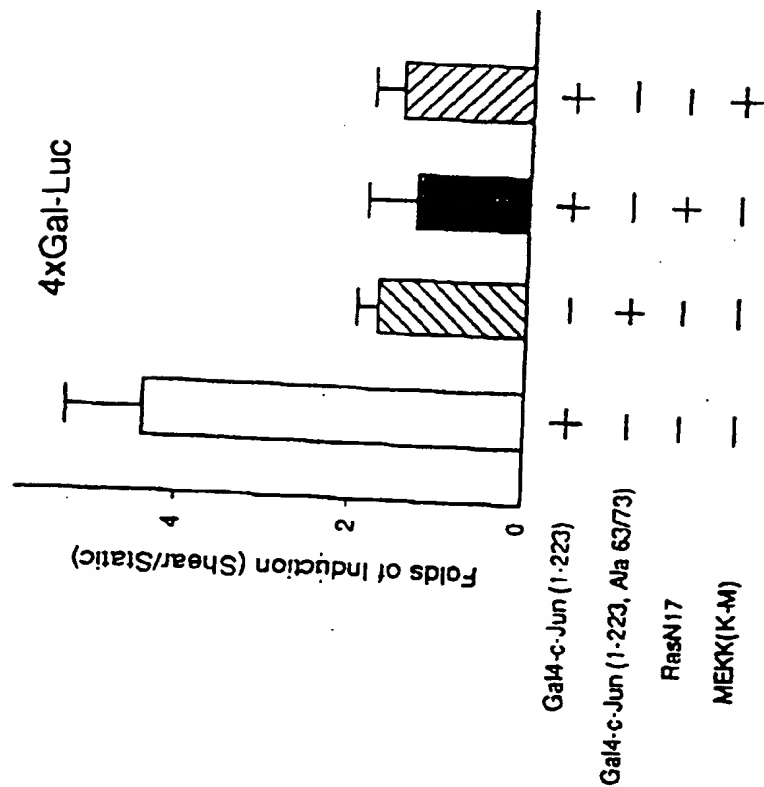


Fig. 5

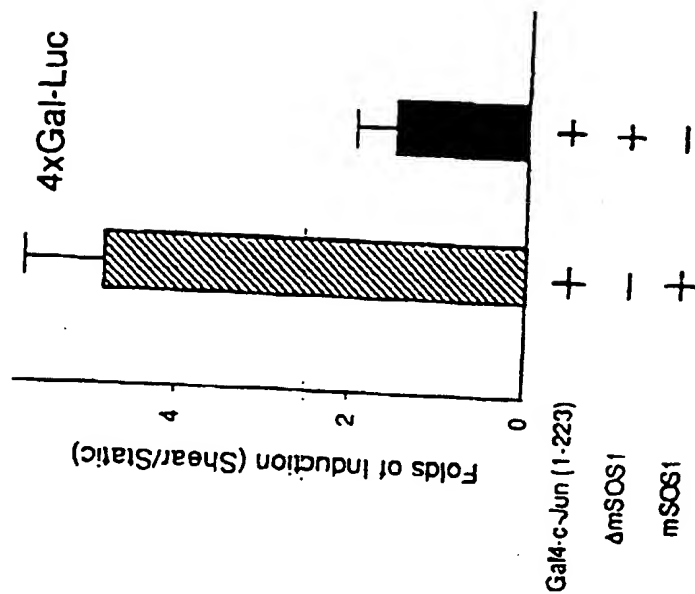
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Fig. 6

A.

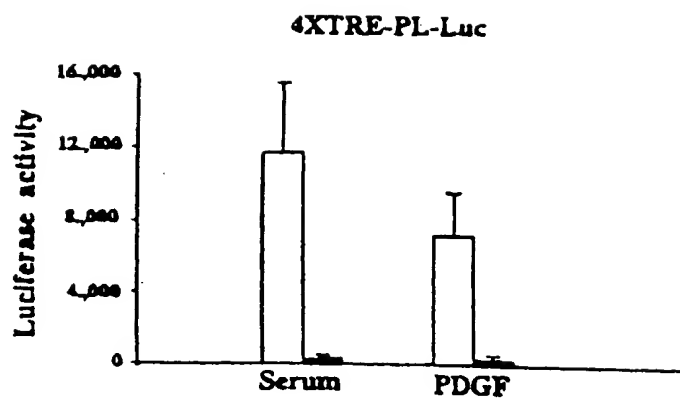


B.



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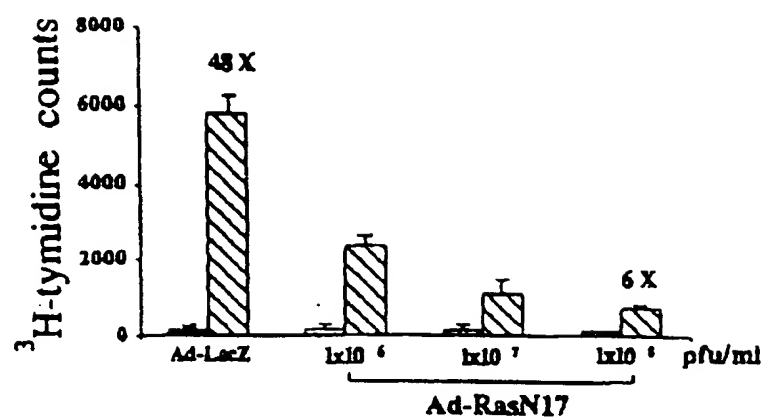
Fig. 7



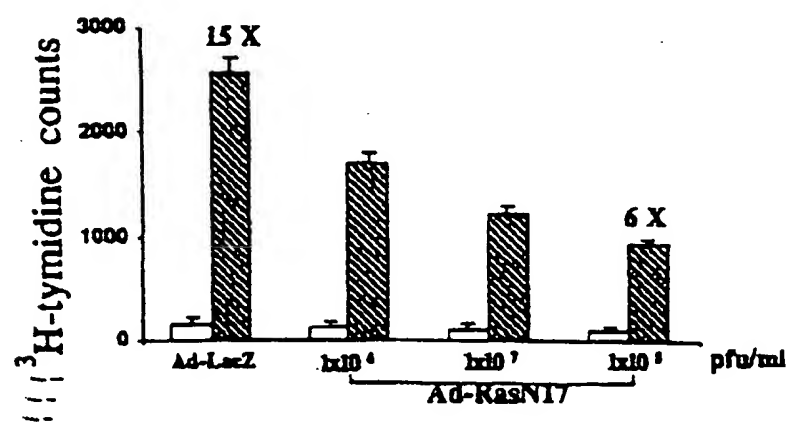
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Fig. 8

A.

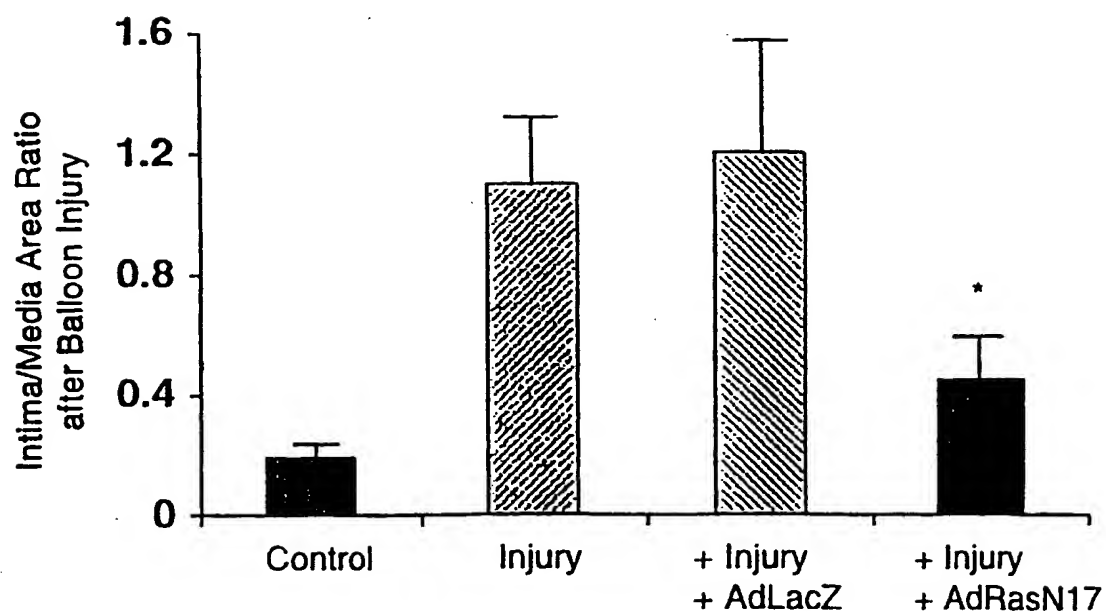


B.



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Fig. 9



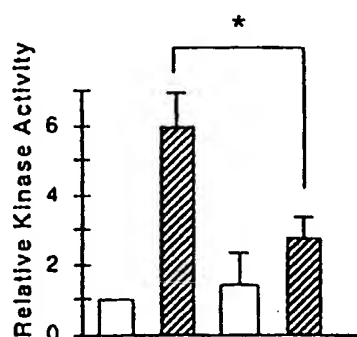


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A.

Fig. 10

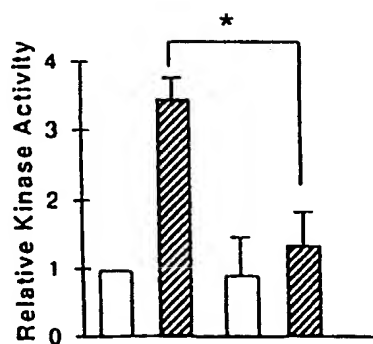
v-src(K295R) - - + +



B.

c-src(K295R) - - + +

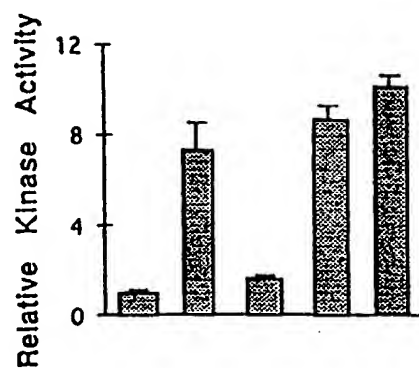
c-src(wt) + + - -



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Fig. 10C

c-src(F527)	-	+	+	-	-
RasN17	-	-	+	-	-
RasL61	-	-	-	+	+
v-src(K295R)	-	-	-	-	+



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/20404

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A61K 31/70; C07H 21/00

US CL : 514/44; 536 23.5, 24.5

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 325, 375; 514/44; 536/23.5, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LI et al. The Ras-Jnk Pathway is Involved in Shear-Induced Gene Expression. Molecular and Cellular Biology. 01 November 1996, Vol. 16, No. 11, pages 5947-5954, see entire document.	1-51
X	INDOLFI et al. Inhibition of Cellular Ras Prevents Smooth Muscle cell Proliferation After Vascular Injury In Vivo. Nature Medicine. June 1995, Vol. 1, No. 6, pages 513-515, see entire document.	1-3, 7, 19, 20-23, 25, 34, 36-43, 50.
X	SKORSKI et al. Growth Factor-Dependent Inhibition of Normal Hematopoiesis by N-ras Antisense Oligodeoxynucleotides. Journal of Experimental Medicine. March 1992, Vol. 175, pages 743-750, see entire document.	20, 21, 33

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"A"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

13 JANUARY 1998

Date of mailing of the international search report

10 FEB 1998

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/20404

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KOHL et al. Protein Farnesyltransferase inhibitors Block the Growth of Ras-Dependent Tumors in Nude Mice. Proceedings of the National Academy of Science, USA. September 1994, Vol. 91, pages 9141-9145, see entire document.	20, 33

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/20404

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS; STN (BIOSIS, CAPLUS, LIFESCI, MEDLINE, INPADOC, WPIDS).

Search terms: fluid, shear, induc?, stress, inhibit?, incompetent, defective, signal?, vascular?, restenosis, stenosis, gene therapy, expression vector, antisense, ras, jnk, jun, src, mekk, fak, ikk, shc, grb2, sos, Chien S, Shyy J Y J.